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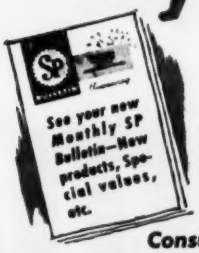

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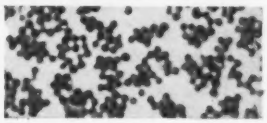
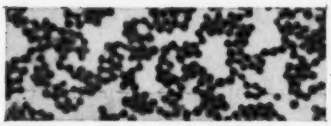
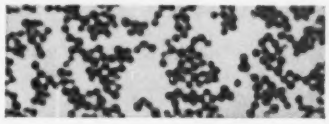
when sensitivity   testing reveals infection due to resistant staphylococci 

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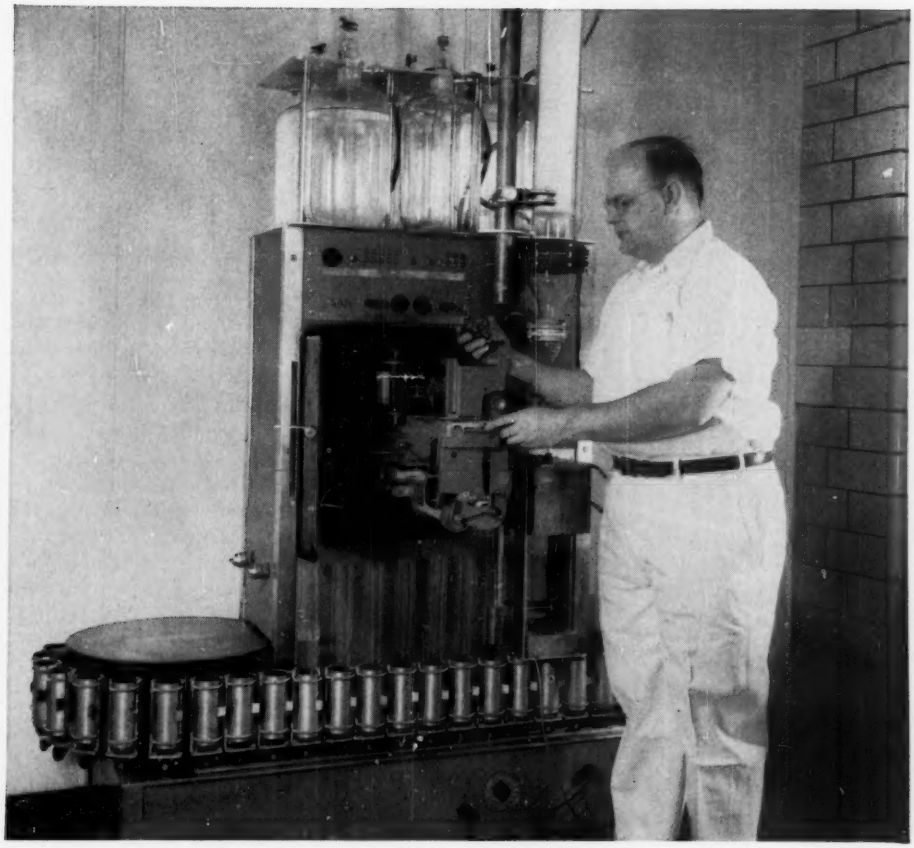

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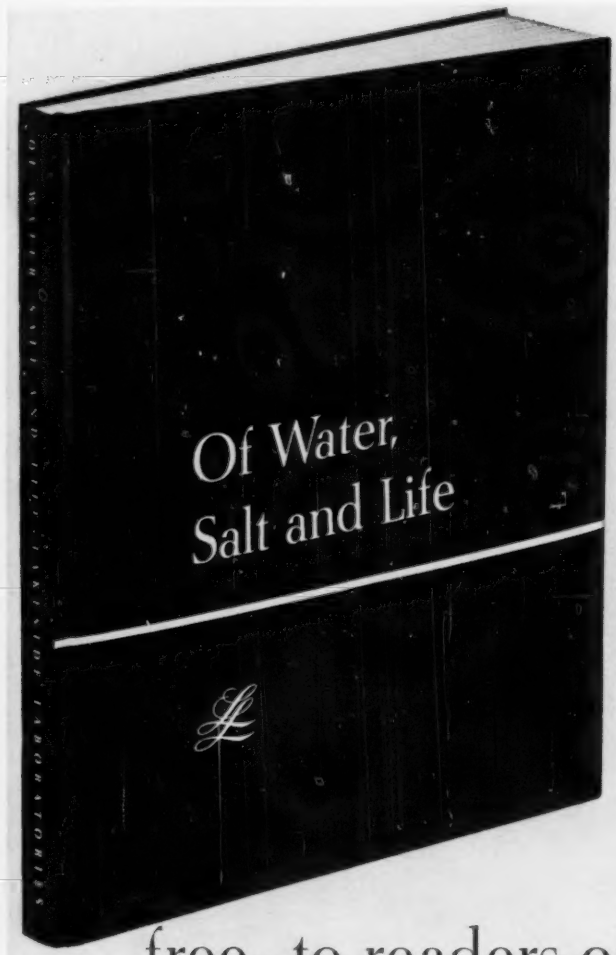

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DESOXYRIBONUCLEASE I ACTIVITY IN PANCREATIC DISEASE[1]

By O. D. KOWLESSAR[2] AND R. K. McEVOY

(From the Division of Experimental Radiology and Surgery, University of Rochester School of Medicine and Dentistry, Rochester, New York)

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The depolymerases (DNase I and II) of desoxyribonucleic acid (DNA) are among the enzymes present in the blood of man and animals (1–3). Since the activity of DNase I in pancreatic tissue is considerably higher than that in other tissues of the body (4), it was postulated by the senior author that an inflammatory or destructive process within the pancreas might result in an alteration of the DNase activity normally observed in the blood. The present investigation was undertaken in order to explore the possibility of using the level of serum DNase I as an index of pancreatic damage. A study of DNase I activity was made on the blood of normal human subjects, of patients with pancreatic and other diseases and of animals with experimentally-induced pancreatitis.

METHODS

The patients included 32 normal subjects, 25 hospitalized patients with various diseases unrelated to the pancreas, and 34 patients suspected of having pancreatic disease and in whom the serum amylase level was 200 units or more. The latter group of patients was subdivided into the groups shown in Tables III and IV on the basis of the laboratory data and the clinical course of their disease including autopsy or operative findings. Single samples of venous blood were drawn from the first and second groups, whereas blood samples were taken every other day from patients with high serum amylase levels.

Edematous pancreatitis was produced in dogs by the following technique. Of five mongrel dogs of both sexes weighing 9.5 to 11 Kg., the pancreatic ducts of three were ligated according to the technique described by Radakovich, Pearse, and Strain (5) and the pathological changes in the pancreas were confirmed by postmortem examination. In the two control dogs, the pancreatic glands were exposed and manipulated but not removed. All the animals were permitted to eat and drink *ad libitum* postoperatively. Blood was withdrawn daily from the femoral vein for three days preoperatively and nine days postoperatively. The serum was separated immediately and assayed for DNase I and amylase.

The following procedure was employed to induce acute pancreatitis in rats. Forty-eight Sprague-Dawley rats of both sexes, weighing 180 to 200 gm., were divided randomly into two groups, one of which was given intraperitoneal injections of one mg. DL-ethionine[3] in aqueous solution at 50° C daily for five days. The control animals were injected intraperitoneally with an equal volume of warm isotonic saline. Both groups were fasted for twelve hours prior to the start of the experiment. The animals were allowed to eat ground Purina fox chow *ad libitum* after the initial period of fasting preceding the first injection. The daily food intake of each animal was weighed and the average amount of food eaten by individual rats in the experimental group was given to each control animal on the following day. Four rats of each group were sacrificed by decapitation on the first, second, third, fourth and fifth days after the first injection. Blood was collected in tubes containing 2 mg. of heparin. The plasma was diluted 1:4 with distilled water and assayed for DNase I and amylase. All the animals were autopsied and the gross findings noted.

DNase I activity was determined by a modification of the method of Allfrey and Mirsky (4). A highly polymerized preparation of sodium desoxyribonucleate obtained from calf thymus according to the method of Kay, Simmons, and Dounce (6) was used as substrate, and tris (hydroxymethyl) aminomethane served as buffer. Enzymatic activity is expressed in micrograms of desoxypentose phosphorus liberated from the substrate per hour of incubation. Serum amylase was determined according to the method of Somogyi (7).

RESULTS

Clinical

The mean and standard error of DNase I activity in the serum of normal individuals and in patients with pancreatic disease and other illnesses are summarized in Table I. The serum DNase values of normal individuals range from .076 to 1.25 μg. of desoxypentose-P per ml. per hour of incubation, with a mean of .46 ± .06 μg. The DNase I activity in the group of randomly selected patients seen in Tables I and II does not deviate

[1] Supported in part by a grant from the United States Atomic Energy Commission.

[2] Present address: The New York Hospital, 525 East 68th Street, New York 21, N. Y.

[3] Nutritional Biochemicals Corp., Inc.

TABLE I

DNase I activity in human sera*

Serum	Number of patients studied	Mean activity and standard deviation of mean
Healthy controls	32	.46 ± .06
Controls with various diseases other than pancreatitis	25	.48 ± .05
Pancreatic diseases†		
A. Edematous pancreatitis	13	.63 ± .05
B. Mumps pancreatitis	2	.33
C. Pancreatic cysts	4	.65 ± .1
D. Pancreatic fistula	1	1.2
E. Carcinoma of pancreas	4	.49 ± .12
F. Acute hemorrhagic pancreatitis	10	7.8 ± .44

* DNase I activity = μg. of desoxypentose-P/hr./ml.
† Enzyme activity expressed as the mean of the average value for each patient within a group.

significantly from the normal level. Table III shows the individual determinations of serum amylase and of DNase I in a series of 23 patients, all of whom were chosen because of elevation of the serum amylase and suspected pancreatic disease. It will be noted that the serum DNase levels of patients with interstitial pancreatitis are significantly higher than those of the controls at the 5 per cent level. Since repeated observation of these patients revealed a fall in DNase I, it appears likely that the initial serum DNase activity was slightly elevated. In contrast to the aforementioned cases, all ten patients with acute hemorrhagic pancreatitis proved at autopsy or exploration showed marked elevation of DNase I activity (Table IV). There was more than a five-fold increase beyond the highest value found in normal subjects.

TABLE II

DNase I activities in sera of patients with various disorders

No.	Patient	Diagnosis	DNase I activity*
1	M. J.	Hernia	0.35
2	J. W.	Hypertension	0.62
3	R. S.	Carcinoma of the breast	0.45
4	A. T.	Pneumonia	0.38
5	R. H.	Hyperthyroidism	0.16
6	L. B.	Infectious hepatitis	0.23
7	M. E.	Acute rheumatic fever	0.19
8	R. M.	Rheumatoid arthritis	0.30
9	O. R.	Tuberculosis of the lung	0.25
10	B. D.	Cerebral vascular accident	0.45
11	F. H.	Infectious hepatitis	0.32
12	D. S.	Nephrotic syndrome	0.40
13	A. R.	Pernicious anemia	0.65
14	A. G.	Sarcoidosis	0.85
15	J. McG.	Myocardial infarction	0.92
16	E. V.	Hodgkin's disease	1.10
17	T. C.	Cirrhosis of the liver	0.85
18	E. C.	Bronchopneumonia	0.32
19	M. C.	Lupus erythematosus	0.65
20	W. S.	Pneumonia	0.15
21	L. M.	Leukemia	0.82
22	N. F.	Pneumonia	0.35
23	C. G.	Cirrhosis of the liver	0.29
24	C. C.	Chronic glomerulonephritis	0.65
25	F. D.	Thrombophlebitis	0.18

* DNase I activity = μg. of desoxypentose-P/hr./ml.

Experimental

The serum DNase I and amylase levels of the operated and control dogs are shown in Figure 1. The DNase values in normal dogs ranged from 4.0 to 5.8 μg. desoxypentose-P per ml. per hour of incubation with a mean of 4.83. The marked temporary increase in serum amylase which occurred on the first and second postoperative day has been described by other authors (8, 9). The DNase I activity of the serum rose gradually to about two and one-half times the preoperative level and returned to normal by the seventh postoperative day.

The DNase I and amylase activities of the plasma of rats given ethionine and of their pair-fed controls are shown in Figure 2. The values in normal rats ranged from 3.2 to 7.7 μg. desoxypentose-P per ml. per hour with a mean of 5.0. A marked increase occurred in the plasma DNase I activity of the ethionine-treated rats, whereas a decrease was noted in the plasma amylase during the first three days of the treatment. The amylase level rose on the fourth and fifth days, a finding reported previously by Henning and Heinkel (10). It should be pointed out that the food intake was reduced markedly on the fourth and fifth days of the ethionine administration. The animals became quite sick as a result of the treatment, and by the fifth day the surviving animals were moribund. The pathological changes in the pancreas and liver corresponded to those described previously (10). The pancreas of the control animals showed no necrosis or loss of basophilia of the acinar cells. There was a slight fatty change in the livers of the pair-fed controls, but no fibrosis or other signs of liver damage.

TABLE III

Serum DNase I and amylase levels† in sera from patients with various pancreatic disorders*

Patient		Day of illness						
		1	2	3	4	5	6	7
Acute interstitial pancreatitis								
M. S.	(Amylase)	30	26	13		6		2
	(DNase)	1.7	1.5	0.9		0.6		0.5
L. L.	(Amylase)	20	18		7			1.5
	(DNase)	0.9	0.9		0.6			0.4
A. D.	(Amylase)	23.6	12	8		1.2		
	(DNase)	0.9	0.5	0.3		0.2		
A. T.	(Amylase)	16	12	5.2		0.8		
	(DNase)	0.7	0.4	0.3		0.2		
S. D.	(Amylase)	20	17	5.5		2.2		
	(DNase)	0.8	0.7	0.6		0.4		
B. S.	(Amylase)	16	8	2		0.8		
	(DNase)	0.7	0.5	0.3		0.3		
S. T.	(Amylase)	17	8.5	5.2		3		1.8
	(DNase)	1.0	0.8	0.7		0.7		0.4
L. R.	(Amylase)	6.8	6	4.2		3	1	
	(DNase)	0.5	0.5	0.5		0.3	0.2	
M. C.	(Amylase)	12	8		4		1.2	
	(DNase)	0.7	0.7		0.6		0.5	
J. B.	(Amylase)	20	18		7	4	1.0	0.8
	(DNase)	0.9	0.9		0.6	0.5	0.3	0.3
N. T.	(Amylase)	15	11		5		2	
	(DNase)	0.8	0.7		0.6		0.5	
F. L.	(Amylase)	12	6	3	1			
	(DNase)	1.1	0.9	0.6	0.6			
C. G.	(Amylase)	8	6	5	2	0.8		
	(DNase)	0.9	0.8	0.6	0.5	0.5		
Mumps pancreatitis								
R. P.	(Amylase)	4.2		2.5		1.2		0.8
	(DNase)	0.4		0.4		0.3		0.3
L. M.	(Amylase)	2.8		1.8		0.8		
	(DNase)	0.4		0.3		0.2		
Pancreatic cyst								
N. T.	(Amylase)	12.5	9	10		4.5		2
	(DNase)	0.8	0.6	0.6		0.6		0.5
B. N.	(Amylase)	16.5	12.5	11		6.5		1.2
	(DNase)	0.5	0.4	0.4		0.3		0.3
J. S.	(Amylase)	22	15	12		5.0		1.0
	(DNase)	0.9	0.9	0.8		0.7		0.5
H. B.	(Amylase)	17	16	9	6	2	0.8	
	(DNase)	1.1	1.0	1.0	0.7	0.6	0.6	
Carcinoma of pancreas								
E. L.	(Amylase)	5.5	4.6			4.8	7.6	
	(DNase)	0.3	0.3			0.3	0.2	
L. B.	(Amylase)	4.5	6.2		5.9	3.5		3.5
	(DNase)	0.5	0.4		0.4	0.3		0.3
V. A.	(Amylase)	6.5	6.2		5.2		4.2	
	(DNase)	0.5	0.5		0.4		0.3	
H. S.	(Amylase)	8.5	6.5		6.2		6.0	5.0
	(DNase)	0.8	0.7		0.7		0.6	0.6

* DNase = μg. of desoxypentose-P/hr./ml.
† Amylase units × 10^2.

DISCUSSION

The results reported in this paper suggest that a marked and protracted elevation of serum DNase I occurs clinically in pancreatic disease only when tissue breakdown is one of the pathologic features —*i.e.*, as in acute hemorrhagic pancreatitis. These findings can be correlated with those noted in ethionine-induced pancreatitis in the rat and with

TABLE IV

DNase I and amylase levels† in sera from patients with acute hemorrhagic pancreatitis*

Patient		Day of illness							
		1	2	3	4	5	6	7	8
L. S.	(Amylase)	23	18	15	12	11	11	16	
	(DNase)	6.2	7.0	7.2	7.2	6.8	7.5	8.2	
T. P.	(Amylase)	15	12	8					
	(DNase)	5.4	6.2	6.2					
R. W.	(Amylase)	18	11		9				
	(DNase)	5.7	6.2		7.5				
T. R.	(Amylase)	9.2		8.7	8.0	8.0			
	(DNase)	7.5		8.2	8.5	8.6			
M. T.	(Amylase)	15		14	12				
	(DNase)	8.5		8.2	8.75				
R. W. D.	(Amylase)	5.5	.95						
	(DNase)	9.7	9.8						
J. H. C.	(Amylase)	24		16	12	14	15		
	(DNase)	9.5		10.2	10.8	10.5	10.2		
A. L. T.	(Amylase)	7.5	6.2	10	13	17	21	18	15
	(DNase)	6.2	7.3	7.5	7.5	8.0	8.0	8.0	11.5
J. W. S.	(Amylase)	12	13.5	16		15		16.7	
	(DNase)	6.5	7.2	7.5		8.0		8.0	
D. H. C.	(Amylase)	18		17		6.5	5.5	5.8	
	(DNase)	5.5		6.8		7.2	7.5	7.5	

* DNase = μg. of desoxypentose-P/hr./ml.
† Amylase units × 10^2.

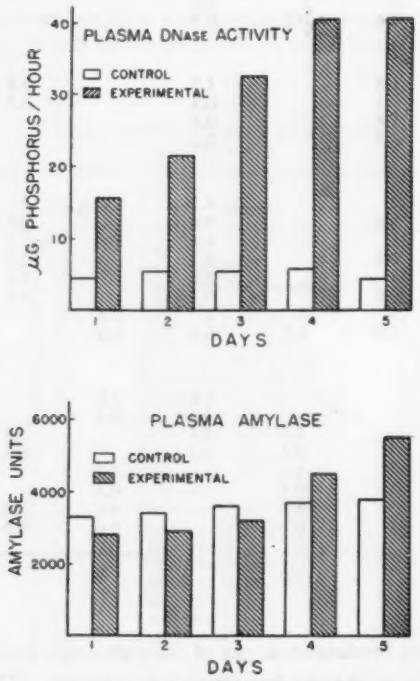

FIG. 1. AVERAGE SERUM DNASE I AND AMYLASE LEVELS IN DOGS WITH LIGATED PANCREATIC DUCTS AND IN SHAM-OPERATED CONTROLS

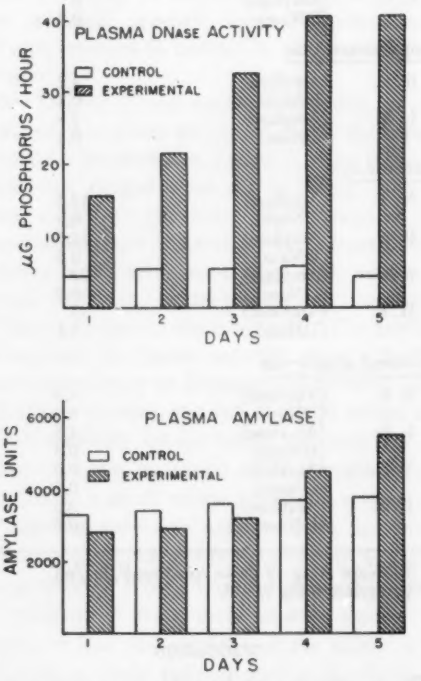

FIG. 2. AVERAGE PLASMA DNASE I AND AMYLASE ACTIVITY IN RATS TREATED WITH ETHIONINE AND IN PAIR-FED UNTREATED CONTROLS

the gradual increase in plasma DNase I activity occurring subsequent to tissue destruction in irradiated rats (11, 12). When pancreatic dysfunction in patients was the result of an inflammatory process not accompanied by necrosis, or when it resulted from operative ligation of the pancreatic ducts in dogs, the DNase activity of the blood increased to approximately twice the preoperative level.

Since this and other studies of serum DNase I activity in patients with a variety of diseases unrelated to the pancreas show normal enzyme activity, it seems likely that the pancreas plays a key role in the production and distribution of DNase I. The high concentration of this enzyme in the pancreatic juice of a dog with a pancreatic fistula (4) supports such a theory, as does the observation in this laboratory of a reduced DNase activity in the duodenal juice of patients with pancreatic fibrosis. Whether DNase I has an exocrine digestive function remains to be established, but its pH optimum (7.6) is compatible with such an action.

Once these findings have been firmly established by more extensive clinical studies, the diagnostic applications are evident. The serum amylase test for pancreatic disease, the laboratory test most extensively used, is subject to limitations (13, 14) and is not specific for pancreatic disease, being abnormal in many other conditions (15–20). Since DNase I is found in high concentrations only in the pancreas and pancreatic juice, alteration in its activity in serum may provide information which will supplement that gained from amylase assay and other studies. In particular, the level of DNase I activity may be of specific value in the diagnosis of necrosis of pancreatic tissue such as is found in acute hemorrhagic necrosis.

SUMMARY AND CONCLUSIONS

1. Serum DNase I was determined in 32 normal human subjects, 25 patients with various diseases unrelated to the pancreas, and 34 patients with pancreatic disease. A marked and protracted elevation in enzyme activity occurred only in patients with acute hemorrhagic pancreatitis. A temporary elevation to the upper limit of normal was noted in patients with edematous pancreatitis.

2. Serum DNase I activity was observed to be temporarily elevated in three dogs after ligation of the pancreatic ducts.

3. Plasma DNase I showed a marked and sustained elevation in rats with ethionine-induced pancreatitis.

4. It was concluded that a marked and sustained rise in blood DNase I activity was noted when there was true necrosis of pancreatic tissue.

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METABOLISM OF 4-C^{14}-TESTOSTERONE IN HUMAN SUBJECTS. I. DISTRIBUTION IN BILE, BLOOD, FECES AND URINE

By AVERY A. SANDBERG[1] AND W. ROY SLAUNWHITE, JR.

(From the Roswell Park Memorial Institute, Buffalo, N. Y.)

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The metabolism of testosterone administered to human subjects was studied soon after the isolation and synthesis of the testicular steroid hormone. In 1939, Dorfman, Cook, and Hamilton (1) and Callow (2) almost simultaneously reported the isolation of androsterone and etiocholanolone in the urine of men receiving testosterone propionate. During the 1940's, Lieberman, Dobriner and their co-workers (3–5) published extensive studies on steroids in human urine related to the metabolism of testosterone. West and his colleagues (6, 7) not only studied the urinary metabolites following the intravenous administration of testosterone (in human serum albumin), but in addition reported studies on the clearance from blood of testosterone and its metabolites (17-ketosteroids). All of the investigations cited were performed following the administration of very large, and hence unphysiological, doses of testosterone.

It was not until methods were developed for the synthesis of labelled testosterone that it became possible to study the metabolism of the hormone administered in physiological doses. Gallagher, Fukushima, Dobriner and their associates were the first to report observations on the metabolism of C^{14} and deuterium labelled testosterone (8–11). The amounts of testosterone administered by these investigators approximated, in most cases, the quantity of hormone calculated to be excreted by the testes. Fukushima, Dobriner, Gallagher, and Bradlow (10, 11) reported that the preponderant part of the administered labelled testosterone appeared in the urine with small amounts in the feces. The authors did not report on the radioactivity in blood.

The biliary excretion of steroids in human subjects has not been studied extensively. Such studies are of importance in the elucidation of testosterone metabolism in man, since it has been demonstrated that following the administration of non-labelled or C^{14}-testosterone to animals (dogs, rats, mice) most of the androgenic material or radioactivity appears in the bile or feces (12–14). In guinea pigs, however, most of the radioactivity following the administration of C^{14}-testosterone has been found in the urine (15). A study of biliary excretion of steroids in human subjects following the oral administration of unlabelled testosterone propionate was reported by Rubin, Dorfman, and Miller (16). No increase in biliary excretion of either 17-ketosteroids or androgens was found. On the other hand, following the administration of estradiol dipropionate they found definite evidence for biliary excretion of its metabolites.

The present paper presents the results of studies on the metabolism of 4-C^{14}-testosterone in 20 adult human subjects of both sexes. The group includes five patients with bile-fistulas, three patients with carcinoma of the prostate, four patients with cancer of the breast, one patient with malignant melanoma, and seven normal subjects. In addition to the urinary and fecal excretion of radioactivity following the administration of 4-C^{14}-testosterone, data are presented on biliary excretion in five patients with bile-fistulas and on the clearance from plasma of free and conjugated radioactive metabolites of 4-C^{14}-testosterone in 17 subjects. In following reports characterization of the metabolites in plasma, bile and urine will be presented.

MATERIALS AND METHODS

4-C^{14}-testosterone (specific activity, 5 μC per mgm.), synthesized according to the method of Fujimoto (17), was obtained from two sources.[2] When the preparations were chromatographed in our laboratory, over 99 per cent of the radioactivity, determined in an Actigraph

[1] Scholar in Cancer Research of the American Cancer Society (1953–56).

[2] We wish to thank Dr. George I. Fujimoto for the 4-C^{14}-testosterone used in the original part of the study. The remainder of the 4-C^{14}-testosterone was secured from Raychem Laboratories, Elmsford, New York.

(Nuclear Chicago), was located in the area corresponding to that of standard testosterone.

The 4-C^{14}-testosterone was dissolved in absolute ethanol (1 to 2 ml.) and diluted with saline (25 ml.) before intravenous (I.V.) injection. The I.V. administration (1 to 5 μC) usually lasted 1 to 2 minutes and the middle of the injection was taken as "zero" time. Blood samples of 30 ml. were drawn 15, 30, 60, 120 and 240 minutes after injection. Heparin was used as an anticoagulant (1 to 2 mgm. per 30 ml. of blood).

Immediately following the withdrawal of blood, the plasma was separated from the red blood cells. The plasma was extracted 3 times with equal volumes of freshly redistilled chloroform and this fraction was labelled as "free radioactive steroids." The residues (I) following chloroform extraction were then extracted twice with large volumes (100 to 150 ml.) of ethanol and discarded. The alcohol was evaporated by an air-stream (at 45° C) and the residue (II) dissolved in water (30 to 50 ml.). The solution was then adjusted to pH 5 ± 0.1 (acetate buffer) and β-glucuronidase added (300 units per ml.). Following incubation for 48 hrs. at 37° C, it was extracted 3 times with freshly redistilled chloroform. The chloroform was evaporated by a stream of air and the residue comprised the "glucuronide fraction." The remaining solution (III) was adjusted to pH 0.8 to 1.0 with 65 per cent sulfuric acid and extracted continuously with ether for 48 hours. This ether extract was called the "sulfate fraction." The aqueous solution (IV) was made 5 per cent with respect to sulfuric acid, refluxed for 30 to 45 minutes, cooled and extracted in a separatory funnel with ether (3 times).

Bile was collected from patients who had undergone cholecystectomy with insertion of a T-tube into the common bile duct. Complete bile collections were assured by a suction apparatus attached to the T-tube, and by the appearance of acholic stools during the period of investigation. The patients had normal liver functions preoperatively and the C^{14}-testosterone was injected at least 3 days postoperatively.

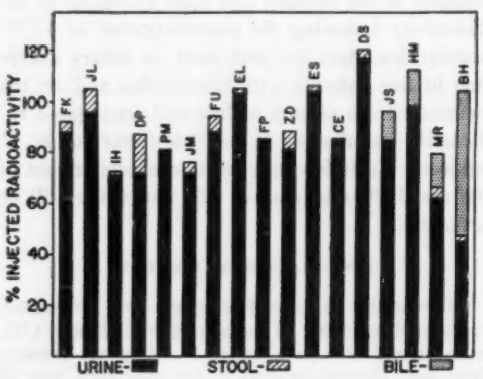

FIG. 1. THE PERCENTAGE OF RADIOACTIVITY EXCRETED IN THE URINE, STOOL AND BILE FOLLOWING 4-C^{14}-TESTOSTERONE ADMINISTRATION

No stool collections were secured from subjects P. M., F. P., and C. E. Bile-fistula patients J. S. and H. M. had negligible radioactivity in the stools.

The bile and urine samples were processed in essentially the same manner as the plasma samples except for the following. The free radioactive steroids were extracted with ether instead of chloroform; following the incubation with β-glucuronidase the steroids were extracted continuously for *48 hours* with ether.

The feces were collected for at least 5 days following the administration of the radioactive testosterone and were mixed with large volumes of ethanol in a Waring blendor and centrifuged. The extraction was performed three times. The alcohol was evaporated and the samples handled similarly to the urine samples.

Radioactivity was measured in a windowless gas flow-counter. A correction for self-absorption was made using suitable calibration curves for inorganic (urine) and organic residues.

The calculation of the total counts in the urine was based on a self-absorption correction curve obtained by the addition of the radioactive steroid to varying amounts of non-radioactive urine. The standard error of these counts did not exceed 5 per cent. The unextracted counts represent the difference between the total counts in the urine and the extractable counts. In those specimens in which the unextractable counts constituted a significant part of the radioactivity (for example, the 4 and 8-hr. specimens of subject J. L. in Figure 2; the 4, 8 and 12-hr. specimens in subject F. K.; the 4-hr. specimen in sub-

TABLE I

Patient	Sex	Age	Diagnosis
D. S.	F	39	Normal
F. K.	F	27	Normal
I. H.	F	38	Normal
S. A.	M	27	Normal
C. P.	M	24	Normal
P. M.	M	25	Normal
D. P.	F	29	Normal
J. L.	F	40	Normal
J. M.	M	73	Carcinoma of prostate with metastases
F. U.	M	66	Carcinoma of prostate, orchidectomy
E. L.	M	75	Carcinoma of prostate
Z. D.	F	46	Carcinoma of breast
E. S.	F	35	Carcinoma of breast
C. E.	F	50	Carcinoma of breast, NPN 69 to 101 mgm. per cent, albuminuria, patient receiving 100 mgm. testosterone propionate 3 times weekly I.M., generalized metastases
F. P.	M	24	Malignant melanoma
H. M.	M	50	T-tube drainage, cholecystectomy for cholecystitis
J. J.	M	38	T-tube drainage, cholecystectomy for cholecystitis
B. H.	F	52	T-tube drainage, cholecystectomy for cholecystitis
W. F.	F	69	T-tube drainage, cholecystectomy for cholecystitis
M. R.	F	60	T-tube drainage, cholecystectomy for cholecystitis, cancer of breast

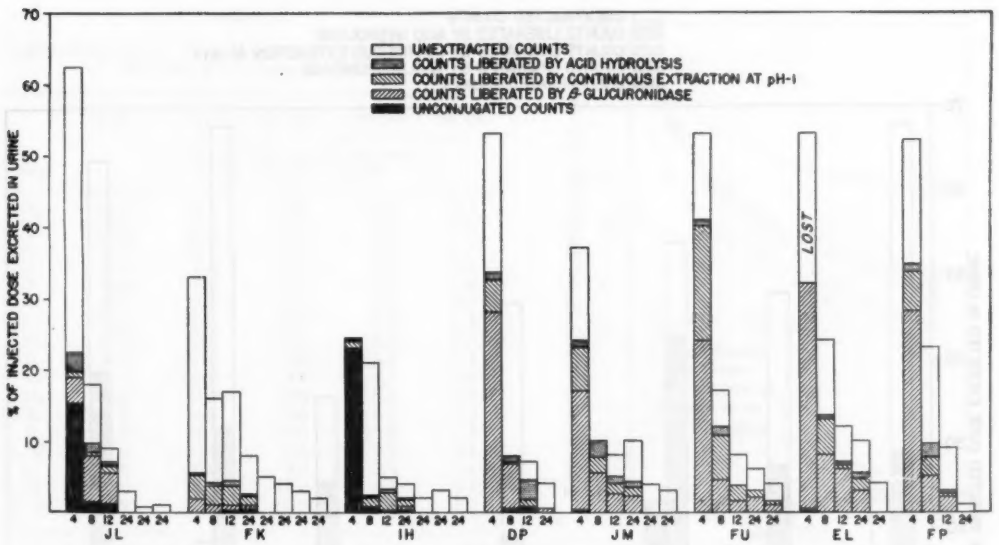

FIG. 2. EXCRETION OF RADIOACTIVITY IN THE URINE FOLLOWING THE INJECTION OF C^{14}-TESTOSTERONE
The numbers refer to the number of hours covered by the period of collection.

ject D. P.; *etc.*) the urine residue, following the various hydrolytic and extraction procedures, was counted. In all cases 80 to 85 per cent of the calculated counts was present in the residues.

RESULTS

In Table I are shown some of the clinical data on the subjects given I.V. 4-C^{14}-testosterone.

In Figure 1 is graphed the percentage of the injected radioactivity excreted in the urine, feces, and bile. The amount excreted in the urine in 48 hours ranged (exclusive of the bile-fistula patients) from 71 to 117 per cent (average 89 per cent). The total radioactivity excreted in the urine of normal subjects did not differ significantly from that of patients with cancer.

In Figures 2 and 3 are shown the percentages of the injected C^{14}-testosterone excreted in the urine during certain time periods following the injection of the steroid. In addition, the amounts of steroid metabolites extractable following various hydrolytic procedures are shown. It is apparent that a preponderant part of the injected testosterone is excreted in conjugated form, primarily as the glucuronide. Except in a few urine samples where, for some reason as yet unknown, large amounts of free metabolites were present, sulfate-conjugated metabolites of testosterone constituted an unimportant part of the radioactivity excreted. A significant part of the radioactivity could not be extracted from the urine even after strong acid hydrolysis.

It is apparent from Figures 2 and 3 that most of the radioactivity following C^{14}-testosterone administration was excreted in the first 4 hours. An exception was noted in C. E., a patient with carcinoma of the breast, with impaired renal and liver functions, and receiving large amounts of testosterone propionate intramuscularly, who excreted the major part of administered radioactive steroid during the second 12-hour period after administration.

The clearance of free radioactive steroids (Figure 4 and Table II) from the plasma of normal subjects and of patients with cancer was rapid. The disappearance curves can be interpreted as indicating two possible rates. The first rate is related to a pool with a half-time of approximately 11 minutes, and the second, slower rate, is related to a pool with a half-time of approximately 100 minutes.

In Figure 5 and Table III are shown the steroid glucuronide plasma levels. Within 15 minutes following the injection, the counts in the plasma glucuronide fraction markedly exceeded those found

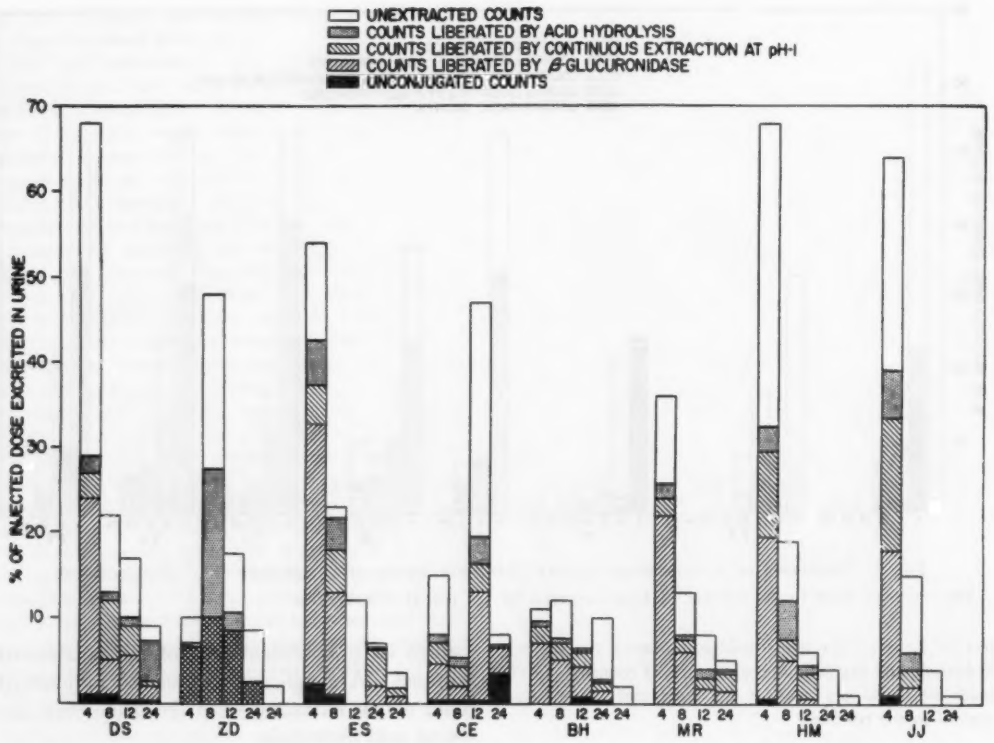

FIG. 3. EXCRETION OF RADIOACTIVITY IN THE URINE FOLLOWING THE INJECTION OF C^{14}-TESTOSTERONE
The numbers along the abscissa indicate the duration of each successive period of collection and are not cumulative.

with the unconjugated steroids. Activity declined slowly thereafter. Since the major identifiable conjugates in the urine are the glucuronides, it is interesting to note that patient C. E., with impaired renal function and receiving testosterone, maintained very high levels of radioactivity in the free steroid and glucuronide fractions during the time of study.

The counts in the sulfate conjugated steroid fraction were about 1/20 of the levels of the glucuronides and essentially parallel to them. Normal subject J. L. had strikingly higher sulfate levels than the rest of the group at 2 and 4 hours. We have no satisfactory explanation for this observation.

The radioactivity extractable from plasma following strong acid and heat hydrolysis was negligible. Similarly, negligible activity was found when the red blood cells were extracted with absolute alcohol following lysis of the cells with water.

Patients with carcinoma of the breast cleared the free steroid radioactivity from the plasma at a somewhat slower rate than the remainder of the group. Since the number of cases studied was rather small, the possible significance of this finding must await confirmation of the observation in a larger group of similar patients.

The amount of radioactivity excreted in the bile is shown in Figure 6. Four of the patients with T-tube drainage excreted 11 to 14 per cent of the radioactivity of the injected C^{14}-testosterone in the bile, with most of the excretion occurring in the first 4 hours. Patient B. H. excreted 56 per cent of the injected radioactivity in the bile, mostly in the first 4 hours. In Figure 3 it can be seen that this patient (B. H.) excreted much less of the radioactivity in the urine during the first 12

TABLE II

Radioactivity levels in free steroid fraction of plasma following injection of 4-C^{14}-testosterone *

Subject	15 min.	30 min.	60 min.	120 min.	240 min.
	% of injected dose per 100 ml. plasma per Kg. body weight × 10^4				
D. S.	41.0	10.0	5.9	0.8	0.3
F. K.	34.0	23.0	11.0	3.9	1.9
I. H.	56.0	26.0	11.0	2.9	1.7
S. A.	11.0	5.1	2.3	1.9	0.4
C. P.	55.0	9.6	4.4	1.7	0.4
D. P.	38.0	15.0	4.3	1.1	0.3
J. L.	74.0	32.0	12.0	3.7	0.8
J. M.	59.0	33.0	13.0	4.3	0.3
F. U.	39.0	23.0	8.0	3.0	0.4
E. L.	45.0	26.0	9.6	5.5	1.6
Z. D.	93.0	47.0	15.0	6.6	3.8
E. S.	47.0	26.0	9.8	4.3	2.3
C. E.	70.0	34.0	25.0	13.0	8.8
H. M.	35.0	17.0	6.4	1.9	0.4
J. J.	27.0	20.0	8.8	3.8	1.4
B. H.	76.0	34.0	17.0	9.3	3.3
M. R.	64.0	23.0	15.0	7.5	3.2

* In Table II the number of counts present in the 120-minute samples was of such magnitude that the counting did not involve a standard error from the mean greater than 5 per cent; in the 240-minute specimens the counting did not involve a standard error from the mean greater than 10 per cent.

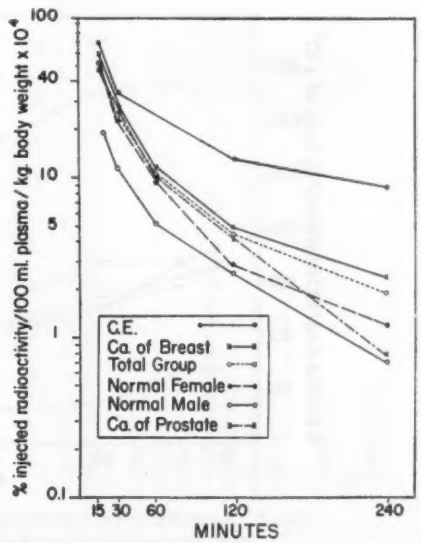

FIG. 4. THE CLEARANCE OF "FREE STEROID" RADIOACTIVITY FROM THE PLASMA FOLLOWING THE I.V. INJECTION OF C^{14}-TESTOSTERONE

hours than any other subject in the whole group. It should be pointed out that although this patient had normal liver function tests preoperatively she developed a gram-negative bacterial septicemia with an ascending cholangitis postoperatively. Even though the C^{14}-testosterone study was performed when the patient seemingly had made a recovery from the infection, it is possible that the infection may have influenced the amount of radioactivity excreted in her bile.

The excretion of free steroids in the bile was negligible, with most of the extractable steroids being glucuronides or sulfates. The bulk of the radioactivity could not be extracted following the hydrolytic procedures used. The amount not extractable from bile was even greater than that from the urine.

The radioactivity excreted in the stools (Figure 7) of non-fistula subjects varied from 2 to 15 per cent (average: approximately 6 per cent). Counts in the free steroid fraction comprised the largest amount. In the patients with T-tube drainage the fecal radioactivity excreted ranged from less than 0.1 per cent to 2 per cent.

DISCUSSION

The nearly quantitative excretion of the radioactivity in the urine following the intravenous injection of 4-C^{14}-testosterone is at variance with results obtained by others when excretion of non-labelled steroid products was studied. This is not surprising, since even with labelled testosterone only about 50 per cent of the radioactivity could be released and extracted following various hydrolytic procedures. In addition, the estimation of the urinary excretion of metabolites following non-radioactive administration depended on biological assay or the determination of 17-ketosteroids. It is known that neither procedure would estimate all the metabolites of testosterone in the urine. Our findings are similar to those of Fukushima, Dobriner, Gallagher, and Bradlow (10, 11), who were able to recover 50 per cent of the administered radioactivity from the urine (as crude, ether-extractable, and neutral metabolites) during the first 24 hours.

Gallagher, Fukushima, Dobriner and their associates (9–11) were able to extract from the urines of their published cases from 50 to 77 per cent of radioactivity in the neutral fractions following intravenous testosterone administration,

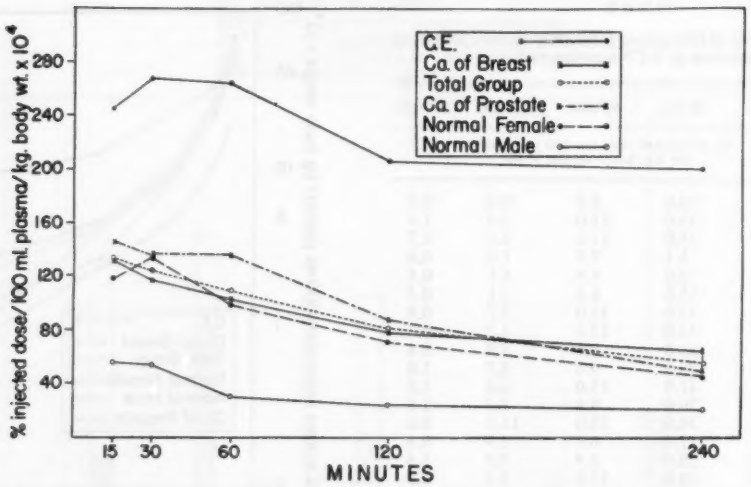

FIG. 5. RADIOACTIVE GLUCURONIDATE STEROID LEVELS IN THE PLASMA FOLLOWING THE ADMINISTRATION OF RADIOACTIVE TESTOSTERONE INTRAVENOUSLY

the amount extractable being dependent on the exact hydrolytic and extractive procedures used (9). It is interesting to note that their highest extractable percentages occurred in those urines on which a repeat β-glucuronidase hydrolysis was performed following an initial incubation with the enzyme and extraction at pH 1. Even though in more than half of our cases 50 to 78 per cent of the administered radioactivity was extracted, it is possible that further incubation with β-glucuronidase would have released additional extractable radioactivity. Furthermore, it is possible that in-

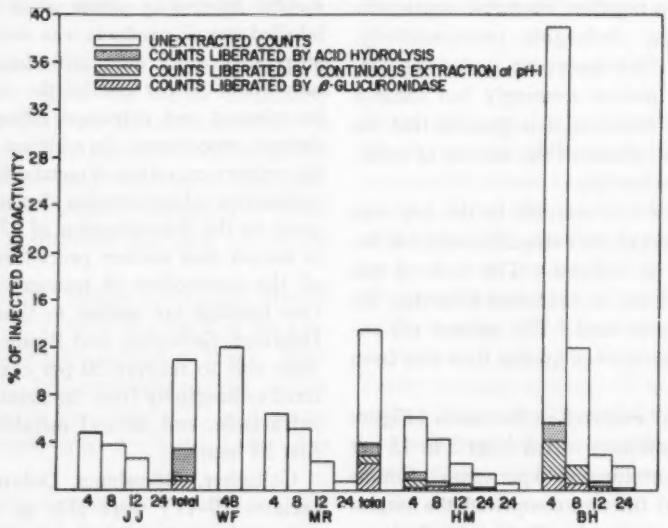

FIG. 6. EXCRETION OF RADIOACTIVITY IN THE BILE OF FIVE PATIENTS

The numbers refer to the number of hours covered by the collection. Note absence of free steroids in the bile.

cubation with β-glucuronidase for longer periods of time, such as employed by Gallagher and associates (120 hours) (9), than used in our experiments (48 hours) would have resulted in a greater percentage of extractable counts in our cases. In addition, the minor discrepancies between our results and those of Gallagher, Fukushima, Dobriner, *et al.* (8–11) may possibly be due to the larger amounts of carrier used (8.4 to 16.0 mgm.) by the above authors and differences in the methods and rates of infusion of the C^{14}-testosterone.

Radioactive testosterone was cleared from the plasma rapidly when compared to the clearance of 4-C^{14}-cortisol (18–20). Within 15 minutes following the injection of the 4-C^{14}-testosterone the radioactivity in the glucuronide fraction reached its peak and was about threefold the level in the "free" steroid fraction. This contrasts with the metabolism of C^{14}-cortisol where the counts in the glucuronide fraction achieve their peak and equal the level in the free steroid fraction in about 120 minutes (19). The glucuronide fraction radioactivity following C^{14}-testosterone reached its peak early and declined slowly thereafter. Since the excretion of radioactive metabolites in the urine during the first 4-hour period was at least 50 per cent of the administered dose, it indicates that testosterone had been taken up by tissues and its glucuronide-conjugated metabolites released slowly over that period. Even though the differences in the methods used make absolute comparison difficult, the clearances of C^{14}-testosterone from plasma and of radioactivity of the conjugates in our studies give distributional findings similar to those of West, Tyler, Brown, and Samuels (7) following the administration of large doses of non-radioactive testosterone.

The free steroids disappeared from the plasma with at least two different rates, an initial fast rate and a subsequent much slower rate. This may indicate that at least two different metabolic pools exist or that the initial rate is that of 4-C^{14}-testosterone clearance from the plasma and the subsequent slower rate that of a "free" steroid metabolite of testosterone. Patient C. E. cleared the C^{14}-testosterone from her plasma slower than any other patient in the group. We believe this was due to impaired liver function in the patient, since it has been shown that the liver plays an important

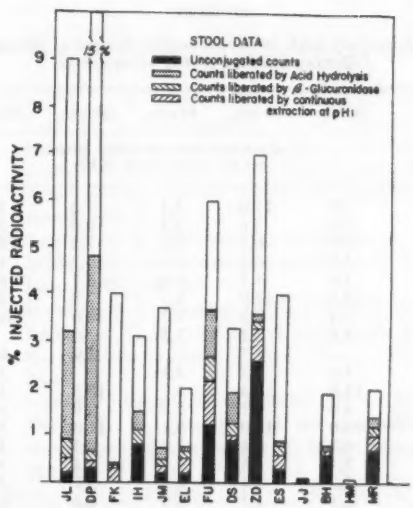

FIG. 7. EXCRETION OF RADIOACTIVITY IN THE STOOLS OF 14 SUBJECTS FOLLOWING THE INJECTION OF C^{14}-TESTOSTERONE

role in the metabolism of free steroids, although other factors in this lady may also have played a part.

The urinary excretion of metabolites following 4-C^{14}-testosterone injection, even though it varied somewhat from subject to subject with respect to the amounts of radioactivity released by the various hydrolytic procedures, showed an essentially con-

TABLE III

Radioactivity levels in steroid glucuronidate fraction of plasma following injection of 4-C^{14}-testosterone

Subject	15 min.	30 min.	60 min.	120 min.	240 min.
	% of injected dose per 100 ml. plasma per Kg. body weight × 10^4				
D. S.	52	53	38	31	23
F. K.	61	111	98	85	38
I. H.	165	165	126	88	78
S. A.	29	21	26	17	11
C. P.	39	36	29	21	18
D. P.	143	157	109	70	44
J. L.	99	98	63	47	21
J. M.	284	231	244	155	
F. U.		79		50	21
E. L.	75	101	85		42
Z. D.	241	200	168	124	103
E. S.	112	108	98	79	76
C. E.	245	268	263	206	200
H. M.	189	144	129	81	37
J. J.	99	101	34	32	27
B. H.	151	128	130	114	85
M. R.	124	111	103	82	44

TABLE IV

Radioactivity levels in steroid sulfate fraction of plasma following injection of 4-C^{14}-testosterone

Subject	15 min.	30 min.	60 min.	120 min.	240 min.
	% of injected dose per 100 ml. plasma per Kg. body weight × 10^4				
D. S.	5.9	11.0	3.1	5.2	8.0
F. K.	5.8	5.6	5.4	6.8	5.7
I. H.	6.9	6.7	5.0	7.2	5.9
S. A.	5.9	6.1	6.2	6.7	4.1
C. P.	1.9	7.7	8.6	6.7	8.0
D. P.	13.0	12.0	9.5	2.8	2.8
J. L.	5.4	7.2	7.7	18.0	34.0
J. M.	4.6	3.2	5.5	2.0	
F. U.		4.7		2.9	0.8
E. L.	1.0	2.5	4.1		3.3
Z. D.	14.0	6.3	4.7	10.5	4.7
E. S.	4.8	7.1	5.5	9.9	3.6
C. E.	13.0	12.0	8.1	11.0	5.1
H. M.	6.2	6.1	7.6	6.3	3.8
J. J.	7.1	5.5	5.4	6.4	3.5
B. H.	7.2	8.4	4.7	3.7	3.5
M. R.	5.1	4.1	4.5	4.6	2.0

sistent pattern. The preponderant part of the radioactivity was excreted in the first 4 hours following injection, except for patient C. E. who had impaired renal function and was receiving large doses of intramuscular testosterone propionate. It is likely that impaired renal function in patient C. E. played a part in the abnormal excretion. The role of large doses of non-radioactive testosterone is difficult to evaluate.

About 50 per cent of the radioactivity was ether or chloroform extractable following the various hydrolytic procedures, with the bulk being in the form of glucuronides. The amount of free steroid excreted in the urine, except for a few specimens in normal subjects I. H. and J. L., was very small when compared to the amount excreted in conjugated forms.

The urinary excretion of radioactivity during the first 4 to 8 hours following C^{14}-testosterone injection was almost double that following C^{14}-cortisol administration and may be related to the faster metabolism of the former steroid (18–20).

The amount of radioactivity excreted in the bile averaged 12 per cent of the injected dose, exclusive of patient B. H. who excreted 56 per cent. This patient had recently had ascending cholangitis and it is possible that her substantial biliary excretion was related to that. The findings in the present study regarding the biliary route of excretion of C^{14}-testosterone and its metabolites differ from those observed in animals. As contrasted to human subjects, animals (rats, mice, dogs) excrete most of the administered testosterone in the bile and very little in the urine (12–14). These differences make comparisons regarding metabolism of testosterone among various species unreliable. Even though Burstein, Ungar, Gut, and Dorfman (15) have reported that most of the radioactivity following administration of C^{14}-testosterone to guinea pigs appeared in the urine, the possibility of substantial biliary excretion of radioactivity with subsequent intestinal reabsorption cannot be excluded.

It has been shown that 1 to 5 per cent of radioactivity following intravenous C^{14}-cortisol administration is excreted in the bile of human subjects (19, 20). On the other hand, at least 30 to 60 per cent of radioactivity following C^{14}-progesterone (21), C^{14}-estrone or C^{14}-estradiol administration is excreted in the bile of man (22).

The fecal excretion of radioactivity in patients without T-tube drainage averaged about 6 per cent. It is apparent that some reabsorption of testosterone metabolites must occur in the gastrointestinal tract. The small amounts (1 per cent) of radioactivity present in the stools of patients with T-tubes may be due either to excretion of radioactivity in gastrointestinal juices or to the escape of small amounts of bile. The fecal excretion of radioactivity in our series is comparable to the results of Fukushima, Bradlow, Dobriner, and Gallagher (11).

Essentially, no unconjugated steroids were present in the bile and the percentage extractable after the various hydrolytic procedures fell far short of the percentages in the urine. On the other hand, the unconjugated (free) steroids constituted a major part of the steroids in the stools. Since it has been demonstrated that human stools contain β-glucuronidase and phenolsulfatase activities (20, 23), it is possible that some of the steroid conjugates originating from the bile are cleaved by the fecal enzymes.

SUMMARY

4-C^{14}-testosterone has been administered intravenously to 20 human subjects. The radioactivity was excreted almost quantitatively in the urine in 48 hours with over 50 per cent in the first 4 hours. About 50 per cent of the radioactivity could be

extracted from urine following different hydrolytic procedures. C^{14}-testosterone was cleared from the plasma rapidly with at least 2 separate rates. These 2 pools had half-lives of 11 and 100 minutes. The conjugated metabolites of C^{14}-testosterone reached their peak in the plasma within 15 minutes following the injection of the steroid. From 12 to 14 per cent of the injected radioactivity was excreted in the bile of subjects with T-tube drainage and only 6 per cent in the stools of patients having no T-tube, which probably indicates reabsorption of steroids from the gastrointestinal tract. The metabolites in bile were in conjugated form, whereas a great part of the radioactivity in the stools was in the free form.

ACKNOWLEDGMENTS

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THE ADAPTATION OF THE NITROUS OXIDE METHOD TO THE DETERMINATION OF RENAL BLOOD FLOW AND *IN VIVO* RENAL WEIGHT IN MAN [1]

By ARCHER P. CROSLEY, JR., JOHN F. BROWN,[2] JOHN H. HUSTON,[3] DEAN A. EMANUEL, HERMAN TUCHMAN,[2] CESAR CASTILLO, AND GEORGE G. ROWE

(From the Cardiovascular Research Laboratory and the Department of Medicine, University of Wisconsin Medical School, and the University Hospitals, Madison, Wis.)

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Since 1945 sodium para-aminohippurate (PAH) (1) has been widely used for the measurement of renal blood flow (2). Since the urinary output of PAH must be measured in this procedure, the errors inherent in urine collection contribute to the error in the determination of renal blood flow. Furthermore, the need to obtain values of urinary concentrations in the calculation of renal blood flow precludes the use of PAH, and therefore of this method, in the study of patients with oliguria or anuria. Recently, Conn, Anderson, and Arena (3) adapted the nitrous oxide method of Kety and Schmidt (4) to the determination of renal blood flow in dogs. The following is a description of the adaptation of this method to the estimation of renal blood flow in man as well as of the utilization of this method for the calculation of renal weight *in vivo*.

METHODS

Five normotensive fasting subjects without clinical evidence of renal disease and seven patients from the medical wards of the University Hospitals were studied. As noted in Table II, the latter presented a great variation in renal functional impairment.

In the five normotensive subjects renal hemodynamics and metabolism were studied by means of standard clearance techniques and right renal vein catheterization (2, 5). In these individuals three fifteen-minute control clearance periods were followed by the determination of renal blood flow by the nitrous oxide method (see below).

In the seven patients demonstrating a variety of renal disease states as well as different degrees of renal functional impairment the method of study was similar to that described for the normotensive subjects with the exception that following the first nitrous oxide determination the study was continued for three additional fifteen-minute periods (45 minutes), at which time a second nitrous oxide renal flow study was performed. This part of the study was designed to determine the reproducibility of the technique.

Renal blood flow by the nitrous oxide method. Renal blood flow was determined by the nitrous oxide method using a modification of the method of Kety and Schmidt (4). A mixture containing approximately 15 per cent nitrous oxide, 20 per cent oxygen and 65 per cent nitrogen was delivered into a 150-liter bag which in turn was attached to a large-bore corrugated rubber tubing through a three-way valve for respiratory gases. The valve was arranged so that the investigators could change the patient's inspiratory gases from room air to the nitrous oxide mixture without the patient's knowledge. Delivery was made to the patient through a rubber mouthpiece attached to the corrugated tubing. The nose was clamped to avoid all leaks. Arterial blood was sampled from the femoral artery through a Cournand needle attached to a plastic tubing which in turn was connected to a manifold consisting of four three-way stopcocks and an end exhaust syringe containing 2 to 5 ml. of heparin. Renal venous blood was obtained from the right renal vein through a cardiac catheter connected directly to a manifold similar to that described for the arterial collection. Since the arterial-venous differences of nitrous oxide in the determination of renal blood flow were very small, it was of great importance to have the dead space on both sides of the blood collection systems as equal as possible, and to have maximal accuracy in the timing and collection of samples as well as in the analysis of specimens of N_2O. The mean difference between duplicate samples of the same blood analyzed for N_2O in this laboratory was .02 vol. per cent (S.D.[4] .02 vol. per cent).

Specimens of blood were collected in oiled, heparinized syringes. Syringes were mounted consecutively on the arterial and venous manifolds with the first specimen most distal to the patient. Prior to each renal blood flow determination, arterial and renal venous blanks were drawn. These should be identical in value as a check of the N_2O analyses.

[1] This investigation was supported in part by grants from the Wisconsin Heart Association, Wisconsin Alumni Research Foundation, and National Heart Institute of the National Institutes of Health (H-1495-C), Public Health Service.

[2] Heart Trainee of the National Heart Institute, U. S. Public Health Service, 1955.

[3] Wisconsin Heart Association Research Fellow.

[4] S.D. = Standard Deviation.

At the time of sampling, blood was drawn into the exhaust syringes at the distal ends of each manifold at a steady rate of one ml. per 5 seconds. The nitrous oxide mixture was turned on after 2½ ml. of blood had been drawn into the exhaust syringes. This arbitrarily assumed volume was equal to 2.5 times the total dead space (one ml.). This volume was assumed to remove from the dead space heparinized blood which was present between determinations or samples. Following the beginning of the administration of the gas mixture, sampling into the exhaust syringes was continued until a total volume of 4 ml. had been obtained. The difference in the volumes of blood in the exhaust syringes between the initial 2½ ml. and the final 4 ml., namely 1½ ml., was estimated to be equal to the time necessary for the nitrous oxide to reach the lung, be absorbed, and be carried in the blood to the orifice of the first sample syringe. These arbitrarily assumed values were not believed to carry particular weight in the determination.

After 4 ml. had been withdrawn into the exhaust syringes, sampling was begun into the first sample syringe at a rate of one ml. per 5 seconds. During the first 1½ minutes of the flow determination three consecutive 6-ml. specimens were obtained at this rate of withdrawal. The fourth 6-ml. specimen was collected from 2 minutes and 45 seconds to 3 minutes and 15 seconds, whereas the fifth specimen consisting of 8 ml. was withdrawn from 4 minutes and 40 seconds to 5 minutes and 20 seconds after the onset of gas administration. At the completion of specimens 3, 4 and 5 the blood in the exhaust syringes was reinjected to clear the system and prevent clotting. Prior to the collection of specimens 4 and 5, the sampling systems were again cleared by drawing blood back into the exhaust syringes at a rate of one ml. per 5 seconds to a total volume of 4 ml. as had been done prior to the first specimen. It is important that the collection into the exhaust syringes proceeds at the same rate as the collection of the specimens in the sample syringes, since the volume of the dead space has already been filled when sampling has begun and this blood constitutes part of the sample submitted for analysis.

The arterial and venous concentrations of N_2O were plotted at the midpoint of the sampling times. The scales used were such that the base line (time) exceeded by two times in length the vertical scale (arterial and renal venous N_2O concentrations). This purposeful distortion of the curves was produced in an effort to gain more accurate readings of the arterial-venous nitrous oxide differences at half-minute intervals instead of minute intervals. The obtaining of half-minute $A\text{-}V_{N_2O}$ differences seemed preferable since the flow rate per unit weight of kidney was rapid and the area between the curves small. Furthermore, since equilibrium by this method is reached in the kidney in 5 minutes, in contrast to a 10-minute period for the brain (4) and myocardium (6), this manner of calculation for renal blood flow provided the same number of $A\text{-}V_{N_2O}$ differences as it is customary to utilize in calculating cerebral or myocardial blood flows. However, this distortion of the curves in the renal method necessitates the multiplication of the final calculated results by two. In these calculations the partition coefficient was assumed to be unity. Such an assumption was based on the findings in the dog kidney where the validity of the N_2O method utilizing this coefficient for measurement of renal blood flow has been demonstrated by comparison with the bubble flow meter (3). More justification for its use may be derived from the similarity of partition coefficients for dog and human brain (4) and myocardium (6).

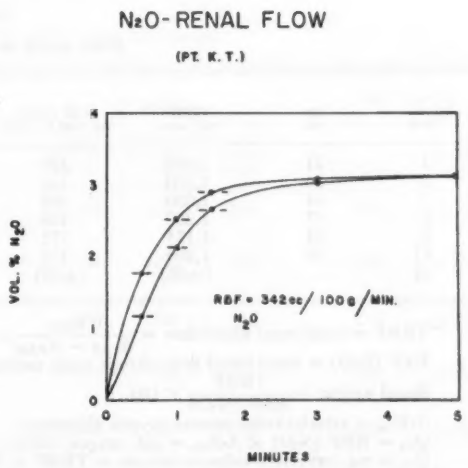

FIG. 1. REPRESENTATIVE NITROUS OXIDE FLOW CURVE

The upper and lower curves represent the arterial and venous, and the nitrous oxide concentrations, respectively. The short horizontal lines denote the 30-second periods of time when the individual samples were obtained during the first 1½ minutes and from which the midpoints were selected (see Methods).

RESULTS

A typical nitrous oxide flow curve is shown in Figure 1.

The data on the five normotensive subjects are presented in Table I. The values for renal blood flow (PAH) ($\bar{x}$ [5] = 1200, S.D. 96 ml. per minute), arterial-renal venous oxygen difference ($\bar{x}$ = 1.3, S.D. 0.2 vol. per cent), and renal oxygen consumption ($RBF_{PAH} \times A\text{-}R_{O_2}$) ($\bar{x}$ = 15.2, S.D. 2.0 ml. per 2 kidneys per minute) are in accord with previous publications (7).

Renal blood flow as determined by the nitrous oxide adaptation equaled $\bar{x}$ = 322, S.D. 59 ml. per 100 G. per minute, whereas renal oxygen consumption, utilizing this method, was $\bar{x}$ = 4.1, S.D. 0.5 ml. per 100 G. per minute.

[5] $\bar{x}$ = mean.

TABLE I

Renal weight in vivo (normal male) *

Pt.†	Age *yrs.*	TRBF *ml./min.*	RBF (N_2O) *ml./100 G./min.*	Renal weight *grams*	A-Ro_2 *vols. %*	Qo_2 *ml./100 G./min.*	Qo_2 *ml./2 Kg./min.*
1	21	1,205	328	367	1.0	3.3	12.1
2	26	1,300	342	380	1.2	4.1	15.1
3	34	1,050	260	404	1.5	3.9	15.8
4	37	1,260	406	310	1.2	4.9	15.1
5	34	1,175	272	432	1.5	4.1	17.8
$\bar{x}$‡	30	1,200	322	379	1.3	4.1	15.2
σ§		(±96)	(±59)	(±46)	(±0.2)	(±0.5)	(±2.0)

* TRBF = total renal blood flow = $\frac{UV_{PAH}}{A_{PAH} - R_{PAH}}$.
RBF (N_2O) = renal blood flow nitrous oxide technique.
Renal weight = $\frac{TRBF}{RBF\ (N_2O)} \times 100$.
A-Ro_2 = arterial-renal venous oxygen difference.
Qo_2 = RBF (N_2O) × A-Ro_2 = ml. oxygen/100 G./min.
Qo_2 = ml. oxygen/2 kidneys/minute = TRBF × A-Ro_2.
† Patient—all were free of organic disease and on psychosomatic service.
‡ $\bar{x}$ = mean.
§ σ = standard deviation.

Renal weight, calculated as the quotient of total renal blood flow (PAH), expressed as ml. per minute, and renal blood flow (N_2O), expressed as ml. per 100 G. per minute, multiplied by 100, was $\bar{x} = 379$, S.D. 46 grams. This figure compares favorably with the kidney weights obtained at autopsy by Wald of $\bar{x} = 323$, S.D. 57 grams (8), and with the mean value of 388 grams given by Lee and Thomas (9) for normotensive males of this age group.

Table II shows initial (control) values as well as values determined 45 minutes later for renal blood flow, determined by the nitrous oxide technique, and for renal weights in seven patients with a variety of renal disease states. The means of the individual ratios relating the initial (control) observations to those obtained 45 minutes later were 1.02 (S.D. 0.11) and 1.02 (S.D. 0.13) for renal blood flow and renal weight, respectively.

DISCUSSION

The assumptions upon which the adaptation of this method to studies of renal blood flow are based have been discussed by Conn, Anderson, and Arena (3) and Conn, Wood, and Schmidt (10) and insofar as possible have been tested in these experiments. In addition it was determined that, even allowing for clearance of the urinary tract "dead space," the urinary loss of nitrous oxide during the five minutes of inhalation was negligible ($< .002$ ml. N_2O). Obviously it has not been possible to check the estimated renal blood flow by the nitrous oxide technique against a direct method, as was done by previous investigators in the dog (3), but the good agreement of the calculated renal weights reported in this paper with those obtained at autopsy (8, 9) supports the validity of the data.

Furthermore, in the calculation of renal weight, particularly in disease states, it must be assumed that: 1) The blood flow per unit weight of kidney (*i.e.*, the N_2O flow) is uniform throughout both kidneys. This might not be true in conditions such as polycystic disease of the kidneys, renal tumors, unilateral renal disease, *etc.* 2) The measurement of renal blood flow by the Fick PAH method represents the *total* renal blood flow through both kidneys. An example for which such an assumption would not be valid is complete unilateral ureteral obstruction. In such an instance the total renal blood flow, and therefore the renal weight, might be in error by 50 per cent of the actual renal weight. 3) The partition coefficient of N_2O remains unity. In these studies this has been assumed to be true. In experiments on dogs (10) in which bilateral renal disease was purposely induced by a variety of methods this coefficient remained unchanged. However, further studies should be performed to evaluate this point either directly, as in patients with renal

TABLE II *

Reproducibility of renal blood flow (N_2O) and renal weight

Pt. Diagnosis	Control			45 minutes			Ratio $\frac{C}{45 \text{ min.}}$	
	TRBF *ml./2 Kg./ min.*	RBF (N_2O) *ml./100 G./ min.*	Renal weight *grams*	TRBF *ml./2 Kg./ min.*	RBF (N_2O) *ml./100 G./ min.*	Renal weight *grams*	RBF (N_2O)	Renal weight
P. S. Chronic pyelonephritis	390	157	248	380	167	228	0.94	1.09
D. S. Diabetic nephropathy	2,030	398	510	1,790	406	440	0.98	1.16
S. R. Essential hypertension	630	336	186	630	348	182	0.97	1.02
L. A. Essential hypertension	1,215	398	307	1,285	394	327	1.01	0.94
K. L. Essential hypertension	1,070	303	353	1,060	324	328	0.94	1.08
M. H. Chronic glomerulonephritis	675	307	220	675	246	274	1.27	0.80
G. G. Chronic glomerulonephritis		377			367		1.03	
$\bar{x}$ =							1.02	1.02
σ =							±0.11	±0.13

* Symbols as in Table I.

disease who are brought to operation for other reasons, or indirectly by comparing *in vivo* and *in vitro* renal weights in such patients as may come to autopsy.

This method presents the following advantages in man over the conventional PAH method: 1) It obviates the necessity for urine collection and its associated error. 2) It may permit the determination of renal blood flow in oliguric states. 3) The entire procedure for the collection of the specimens for subsequent nitrous oxide analyses can be performed in five minutes, thereby allowing a more "instantaneous" measurement of flow than other methods. Such a factor becomes important in calculations of renal oxygen consumption. 4) The technique and materials quantitatively require no greater skills or costs than the PAH method. 5) It provides a method for the determination of renal oxygen consumption in terms of ml. per 100 G. per minute *in vivo* which should lend itself to comparison with renal oxygen consumption *in vitro*.

The disadvantages are those which have been found to hold for the adaptation of this method to the study of cerebral (4) and coronary (11) blood flows.

SUMMARY AND CONCLUSIONS

1. The adaptation of the nitrous oxide technique to the determination of renal blood flow in man is presented.

2. In five healthy male subjects renal blood flow (N_2O) was found to be $\bar{x} = 322$, S.D. 59 ml. per 100 G. per minute.

3. A comparison of calculated renal weights, utilizing this method and total renal blood flow ($\bar{x} = 379$, S.D. 46 grams), with those obtained by others at autopsy ($\bar{x} = 323$, S.D. 57 grams and 388 grams) (8, 9) was favorable and adds validity to the method.

ACKNOWLEDGMENTS

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TOTAL FREE TOCOPHEROLS IN THE SERUM OF PATIENTS WITH THYROID DISEASE[1]

By SHOLEM POSTEL

(From the Department of Medicine of the Harvard Medical School and the Medical Services of the Massachusetts General Hospital, Boston, Mass.)

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The extent of muscle disease encountered at a given level of clinical hyperthyroidism varies from mild subjective weakness to severe disabling myopathy (1). Inconstancy and variable severity of muscle involvement point to some factor apart from the thyrotoxic state *per se* in its genesis. The possibility that exhaustion of tocopherol stores is the critical variable is raised by analogy between the dystrophic morphologic changes, increased oxygen consumption, hypercreatinuria, and depletion of muscle creatine which accompany thyroid hormone excess in animals and man (2–6), and similar alterations due to tocopherol deficiency in rodents (7–11). The mechanisms of these changes are not established, but there is evidence that in both hyperthyroidism (12–14) and tocopherol deficiency (15, 16) impairment of high energy phosphorylations results in uncontrolled release of energy derived from biological oxidations.

The possibility of tocopherol depletion in thyrotoxicosis is supported by evidence of accelerated turnover of other vitamins (17), cholesterol (18), and calcium (19) in hyperthyroidism. Increased tocopherol requirement in the hyperthyroid state is suggested by susceptibility of chicks given excess thyroid hormone to vitamin E deficiency (20). Of further interest are the reports of prevention of thyroxine induced creatinuria in rats (21) and children (22) by α-tocopherol. These considerations stimulated the present survey of the concentration of tocopherols in sera of patients with thyroid disease.

METHODS

Subjects. Observations were made on 115 persons classified as follows:

a. Twenty-nine consecutive patients in whom the diagnosis of Graves' disease was made on clinical grounds and supported by measurement of basal metabolic rate, 24-hour thyroidal uptake of radioactive iodine and, in most cases, determination of the serum concentration of protein bound iodine.

b. Five patients with active Graves' disease by the above-noted criteria who received specific antithyroid therapy within two weeks prior to the first tocopherol determination.

c. Eighteen consecutive patients who were clinically hypothyroid and in whom the above-mentioned tests of thyroid function supported the diagnosis. This group includes one patient receiving methimazole, two patients with spontaneous primary hypothyroidism, and fifteen who developed myxedema after I^{131} treatment of hyperthyroidism or, in one case, heart disease.

d. Thirty random normal subjects, judged to have no significant medical or surgical illness by history, physical examination, and routine laboratory screening consisting of Hinton test, hemoglobin determination, chest roentgenogram, and urinalysis.

e. Thirty-three individuals in the euthyroid state but not otherwise normal. Nineteen had received treatment for hyperthyroidism in the past, one had hepatic cirrhosis, one Hodgkins' lymphoma, one a nodular goiter, and one was in the terminal phase of amyotrophic lateral sclerosis. Three members of this group had diabetes mellitus, and the remaining seven coronary artery or hypertensive vascular disease. In calculating the mean serum tocopherol concentration of the overall euthyroid group, *i.e.*, normal controls plus euthyroid patients with various disorders, the ten patients with diabetes and vascular disease were excluded because they had obviously high levels.

Characterization of the major groupings as to age, sex, height, and weight is summarized in Table I. With the exception of eight thyrotoxic patients and two hypothyroid ones who were hospitalized, all subjects were ambulatory and on self-determined diets.

Tocopherol determinations. The concentration of total free tocopherols in serum was measured by the method of Quaife and Harris (23) as modified by Quaife and Biehler (24). It consists of hexane extraction of 5 ml. of alcohol precipitated serum, palladium catalyzed hydrogenation of the extract for one minute at 20 pounds pressure, and a timed determination of the reduction of ferric ion using the color of the iron α-α-dipyridyl complex as indicator. This method, though not specific, yields results which agree to within 5 per cent with bioassays (23). In the present study, duplicate analyses were made on different days with a simultaneous standard curve accompanying each group of 10 unknowns. The

[1] Supported in part by a grant from the United States Public Health Service (A-849).

TABLE I

Sex, age, height, and weight of subjects grouped according to thyroid status

Group	Number	% Males	Age (*years*) Mean	Age (*years*) Range	Mean height *cm.*	Mean weight *Kg.*
Hyperthyroid	29	7	43.7	(18–77)	159.0	53.9
Normal	30	33	42.7	(19–68)	160.8	66.1
Overall euthyroid	53	25	44.9	(19–75)	158.7	64.0
Hypothyroid	18	11	49.5	(28–73)	158.2	61.6

first 53 consecutive duplicate samples deviated 6.2 ± 4.4 per cent from the average of the two samples. The per cent, as well as absolute differences, tended to be greatest for high values, particularly those over 2.0 mg. per cent. Differences between the first samples and their duplicates analyzed after one to 77 days in the frozen state were not significant. Recovery of 10 to 60 μg. of dl-α-tocopherol from serum or reconstituted dried human plasma in 16 determinations was 97.2 ± 3.1 per cent. Hydrogenation of vitamin A and β-carotene was complete in 15 seconds. Crystalline cholesterol in amounts equivalent to serum concentrations of 0 to 1,200 mg. per cent did not modify the 15-second color density of the tocopherol standards. Iodide in concentrations of 1,000 μg. per cent modified the standard curve, but 100 μg. per cent did not. Also, I^{131} labelled sodium iodide added to serum was not extracted into the hexane solvent. Thyroxine in concentrations up to 100 μg. per cent did not modify the color reaction.

Miscellaneous determinations. Basal metabolic rates were determined by the thyroid laboratory technicians using a Roth-Benedict apparatus. Protein bound iodine was determined by the method of Barker (25). The uptake of radioactive I^{131} twenty-four hours after a 10 to 20 micro-

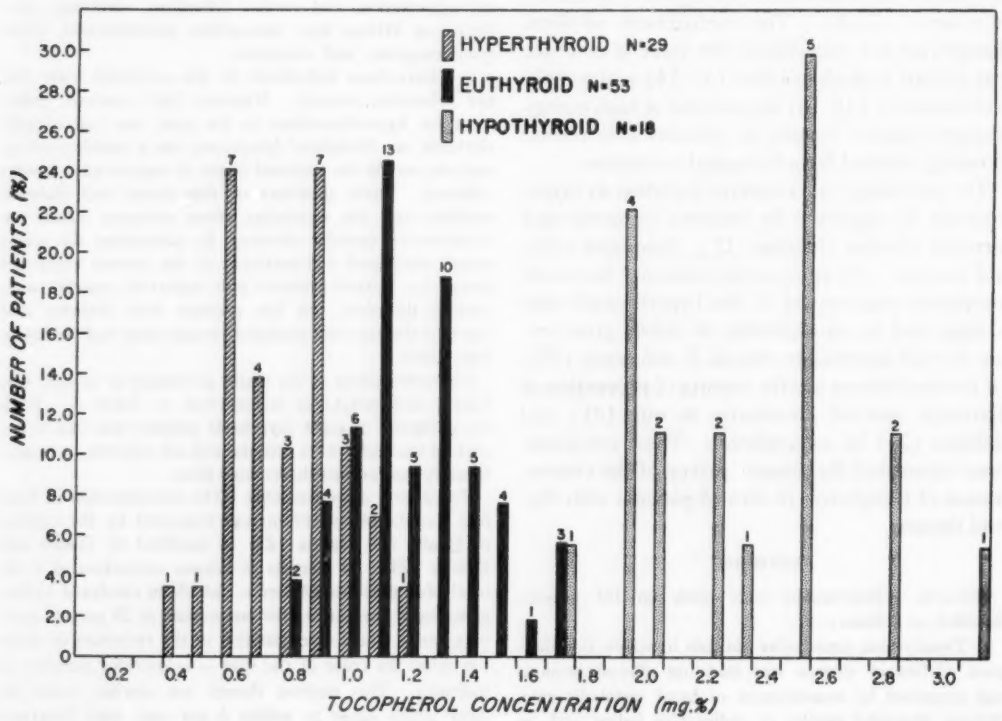

FIG. 1. FREQUENCY DISTRIBUTION OF THE CONCENTRATION OF TOCOPHEROLS IN THE SERUM OF PATIENTS GROUPED ACCORDING TO THEIR STATE OF THYROID FUNCTION

Class intervals equal 0.1 mg. per cent.

curie dose was determined with 3-scintillation detectors placed on a 30 cm. diameter circle. The concentration of cholesterol in serum was determined in the hospital laboratory using the method of Bloor and Knudson (26). Blood samples for tocopherol and cholesterol determinations were drawn at random times in view of the apparent constancy of cholesterol (27–29) and tocopherol (30) levels throughout the day. The symbol ± precedes the standard deviation within the observed population. A statement of significance implies a P value of less than 0.001. Linear correlations of two variables are based on Pearson's product moment coefficient.

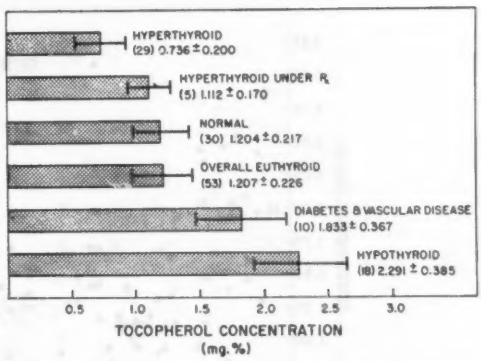

FIG. 2. THE MEAN CONCENTRATION OF TOCOPHEROLS IN THE SERUM OF INDIVIDUALS GROUPED ACCORDING TO METABOLIC STATE

The distance between the two short vertical bars represents, in each case, two standard deviations.

RESULTS

1. *Distribution of serum tocopherol concentrations in hyperthyroidism and hypothyroidism*

The mean tocopherol concentration in 29 patients with active untreated Graves' disease was 0.74 ± 0.20 mg. per cent, compared with a mean value of 1.20 ± 0.22 mg. per cent for the 30 normals and 1.21 ± 0.23 mg. per cent for the group of 53 patients in the euthyroid state. The 18 hypothyroid patients presented with a mean level of 2.29 ± 0.38 mg. per cent. The differences between the hyperthyroid patients, hypothyroid patients and euthyroid group are all significant (P = < .001). The frequency distribution of tocopherol levels among these 100 patients is given in Figure 1, and the mean concentrations for the various populations in Figure 2. Five patients with active Graves' disease who had received treatment either in the form of iodides or antithyroid drugs within two weeks of the initial tocopherol determinations had serum tocopherol concentrations which were not significantly different from the euthyroid group.

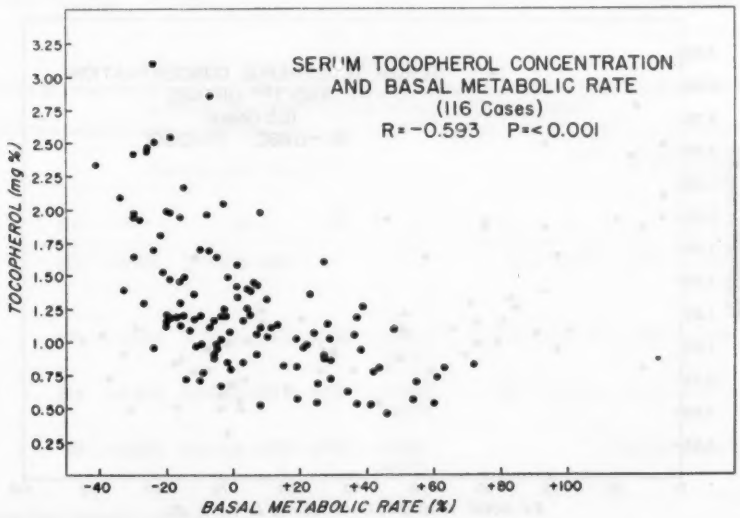

FIG. 3. RELATIONSHIP BETWEEN CONCENTRATION OF TOCOPHEROLS IN SERUM AND BASAL METABOLIC RATE

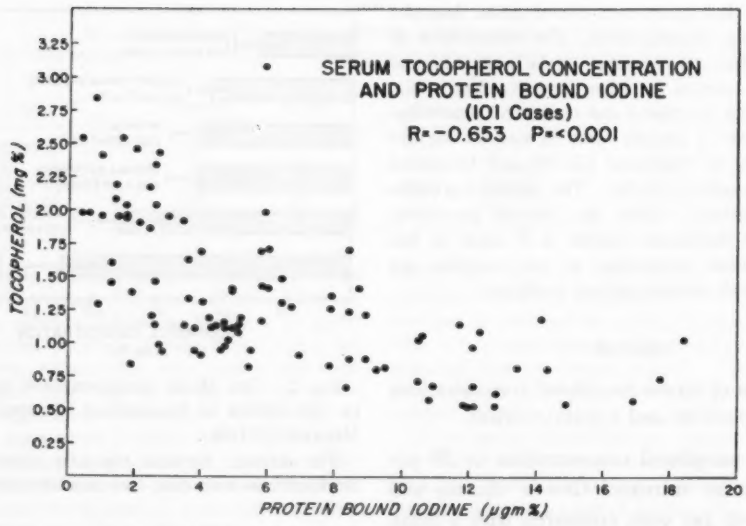

FIG. 4. RELATIONSHIP BETWEEN CONCENTRATION OF TOCOPHEROLS AND PROTEIN BOUND IODINE IN SERUM OF SUBJECTS WITH AND WITHOUT THYROID DISEASE

2. Correlation between serum tocopherol level and metabolic state

Having defined low and high serum tocopherol levels in patients with hyperthyroidism and hypothyroidism, respectively, an attempt was made to appraise the extent to which the tocopherol concentration parallels the metabolic state of the patient. In the absence of an accurate direct index of level of metabolism, the correlations between tocopherol level and basal metabolic rate, serum protein bound iodine (PBI) and 24-hour radioactive iodine uptake, respectively, were examined. Figure

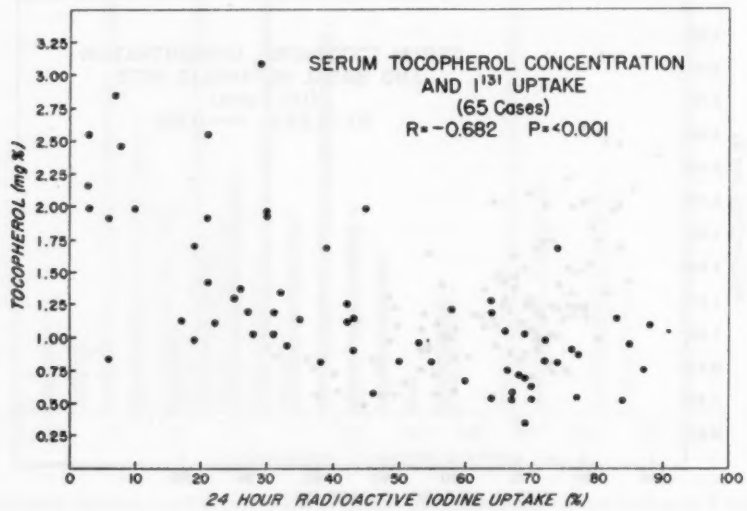

FIG. 5. RELATIONSHIP BETWEEN CONCENTRATION OF TOCOPHEROLS IN SERUM AND THE 24-HOUR THYROIDAL UPTAKE OF I^{131}

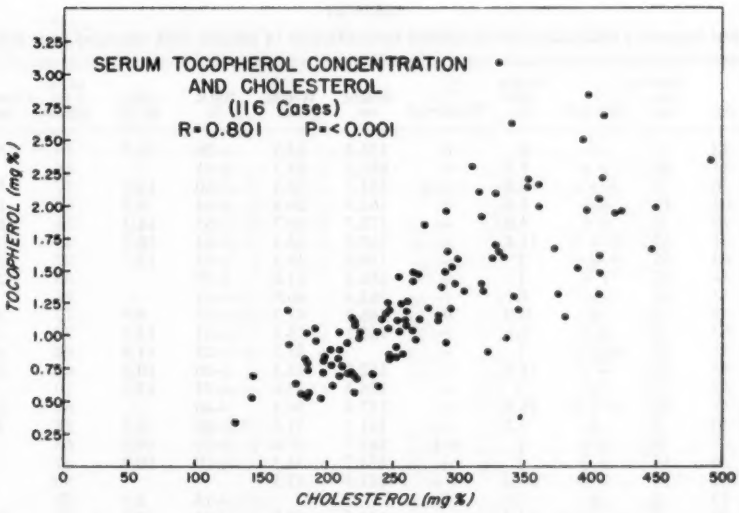

FIG. 6. RELATIONSHIP BETWEEN THE CONCENTRATIONS OF TOCOPHEROLS AND CHOLESTEROL IN THE SERUM

3 is the scattergram of all of the 116 instances in which tocopherol level and BMR were determined on the same day. The coefficient of correlation was − 0.593. The coefficient of correlation for 116 paired values of tocopherol and PBI was − 0.653 (Figure 4), and for 65 pairs of simultaneous tocopherol determinations and 24-hour radioactive iodine uptake − 0.682 (Figure 5). "Z" transformations of these coefficients indicate a probability of less than 0.001 in each case.

3. *Correlation between tocopherol and cholesterol concentrations in serum*

The fat solubility of tocopherols and the well established inverse relationship between metabolic level and serum lipid concentration, particularly

TABLE II

Correlation of serum tocopherol and serum cholesterol concentrations compared with correlation of serum tocopherol concentration with indices of thyroid function

	Maximum pairs (all subjects)					Hypo- and hyperthyroid subjects under treatment				
	No.	"r"*	Z†	$\frac{\Delta Z}{SE\Delta}$	P	No.	"r"	Z	$\frac{\Delta Z}{SE\Delta}$	P
Tocopherol and cholesterol compared with:	116	+0.801	1.102 ± .0941			56	+0.790	1.071 ± .1374		
Tocopherol and PBI	101	−0.653	0.780 ±1010	2.331	<0.02 >0.01	56	−0.717	0.902 ± .1374	0.871	>0.33
Tocopherol and BMR	116	−0.593	0.682±.0941	3.158	<0.005 >0.001	56	−0.736	0.941 ± .1374	0.205	>0.50
Tocopherol and tracer ‡	65	−0.682	0.833±.1270	1.702	<0.10 >0.05			Not analyzed §		

* Pearson's product moment coefficient.
† Fisher's "Z" transformation.
‡ Twenty-four-hour uptake of I^{131}.
§ Tracer data excluded because patients receiving treatment.

TABLE III

Clinical and laboratory data and serum tocopherol concentrations in patients with untreated toxic diffuse goiter

Pt.	Sex	Age	Duration *mos.*	Severity*	Weight loss *Kg.*	Weakness†	Height *cm.*	Weight *Kg.*	BMR *%*	PBI *μg. %*	24-hr. I-131 uptake	Cholesterol *mg. %*	Tocopherol *mg. %*
A. F.	F	43	5	+	0	0	156.2	52.9	+28	11.7	83	380	1.14
B. G.	F	41	30	++	8.2	+	166.5	55.7	+61		66		0.74
C. E.	F	36	9	+++	6.8	+++	154.7	36.1	+60	14.9	77	183	0.54
C. T.	M	64	12	++	5.4	+	162.8	59.8	+44	9.2	55	198	0.80
C. B.	F	22	4	++	4.0	+	172.3	69.7	+63	14.3	74	183	0.81
C. T.	M	31	12	+++	11.4	+	168.8	48.4	+54	16.9	46	210	0.57
D. M.	F	63	36	+++	‡	+	150.5	49.2	+42	17.7	87	211	0.75
D. A.	F	54	24	++	‡	+	154.3	41.8	+29		68	257	0.86
D. L.	F	52	24	+	4.1	+	163.4	66.9	+21		85		0.96
D. M.	F	35	?	+	0.0	0	146.0	47.2	+27	8.9	77	321	0.87
F. A.	F	52	5	++	3.6	+	165.1	75.7	+37	12.1	70	185	0.52
F. D.	F	31	12	+++	‡	+		57.2	+25	11.8	64	182	0.53
F. M.	F	53	36	++	21.4	+	157.1	45.1	+36	10.6	66	264	1.04
G. C.	F	52	6	+	‡	+	156.5	55.6	+22	12.1	79		0.98
H. B.	F	37	10	+++	16.8	+	157.4	56.1	+46		69	130	0.34
H. M.	F	40	3	+	9.1	+	161.1	51.8	+48	12.3	88	221	1.09
H. D.	F	31	6	++	‡	++	162.7	47.6	+29	10.5	68		0.72
H. M.	F	38	12	++	‡	+	154.7	34.1	+19	10.8	67		0.57
K. G.	F	67	24	++	31.8	++	161.5	47.5			90		0.82
K. B.	F	25	3	+	3.6	0			+15	5.4	50		0.82
K. A.	F	24	5	+++	22.2	+	161.7	67.9	+41	12.0	84	137	0.52
L. E.	F	38	6	++	‡	+		43.2			78		0.64
L. A.	F	61	18	+++	36.4	+	157.4	42.8	+72	13.4	72	247	0.82
M. N.	F	18	24	++	‡	+‖		76.2	+55	9.8	51	169	0.48
M. E.	F	77	12	+++	‡	+++		40.0		12.1	69	143	0.68
P. M.	F	49	12	++	0.0	++	165.0	89.0	− 4	8.3	60	222	0.67
R. I.	F	53	12	+	4.1	+	156.4	53.9	+34	12.8	70	238	0.62
R. D.	F	52	84	+++	0.0	+++	158.0	47.3	+ 8	12.8	67	195	0.52
S. H.	F	37	12	+	§	+	151.3	52.9	+19	10.5	69	215	0.94

* One plus: mild; two plus: moderately severe; three plus: severe.

† 0: weakness not a significant symptom; +: weakness present but not a dominant feature; +++: weakness subjectively and objectively marked and labelled "thyrotoxic myopathy."

‡ Lost weight, but amount unspecified.

§ Gained 3.2 Kg.

‖ Atrophy of humeroscapular muscles.

cholesterol, prompted inquiry into the manner in which tocopherol and cholesterol concentrations were correlated. The coefficient of correlation was + 0.801 for 116 paired values spanning a range of 130 to 492 mg. per cent for cholesterol and 0.34 to 3.07 mg. per cent for tocopherol (Figure 6). This degree of correlation is greater than for maximum paired correlations of serum tocopherol concentration with the PBI, BMR, and possibly the 24-hour thyroidal uptake of I^{131} (Table II). In patients with hyperthyroidism and hypothyroidism under treatment, 56 sets of simultaneous tocopherol, BMR, PBI, and cholesterol determinations disclosed no significant difference between the correlations of tocopherol concentration with each of the other variables (Table II).

4. *Correlation between serum tocopherol concentration and obesity or leanness*

A critical difference between thyrotoxic, euthyroid, and hypothyroid groups is the body weight, thyrotoxic subjects generally being underweight, hypothyroid patients tending to obesity. To test the bearing of weight status on tocopherol levels, members of the euthyroid group were ranked as to per cent departure from standard weight (31, 32) for the given height, neglecting age. The range of deviation from standard weight was − 29 per cent to + 97 per cent. Rank correlation with tocopherol concentration was not significant ("r" = + 0.173), indicating that body weight does not in and of itself account for the group differences. In addition, ten subjects presenting a clinical picture suggestive of possible thyrotoxicity but shown by test to be in a euthyroid state, had a mean tocopherol concentration of 1.240 ± 0.32 mg. per cent. This group included one patient with a 30 Kg. weight loss achieved by an 800 to 1,000 calorie diet over a 19-month period. At the end of this period, the tocopherol level was 1.07 mg. per cent. Other cases in point are those of an uncontrolled diabetic who lost 9.1

TABLE IV

Response of serum tocopherol concentrations to treatment of hyperthyroidism

Pt.	Rx.	Interval since Rx. (*weeks*)	Status* post-Rx.	BMR % Pre-Rx.	BMR % Post-Rx.	PBI mg. % Pre-Rx.	PBI mg. % Post-Rx.	Cholesterol mg. % Pre-Rx.	Cholesterol mg. % Post-Rx.	Tocopherol mg. % Pre-Rx.	Tocopherol mg. % Post-Rx.
B. G.	I-131	11	H	+61	−15		2.4		360	0.74	2.16
C. T.	MET†	7	H	+54	+27	16.9	1.3	210	334	0.57	1.60
D. A.	I-131	7	H	+29	−21		2.3	257	390	0.86	1.51
H. D.	$KClO_4$	13	H	+29	−22	10.5	1.0		423	0.72	1.95
H. M.	MET	15	H	+19	−16	10.8	1.3		254	0.57	1.45
P. M.	I-131	17	H	− 4	−24	8.3	4.0	222	327	0.67	1.68
C. B.	MET	7	E	+63	+ 3	11.7	7.3	183		0.81	1.16
C. E.	I-131	30	E	+60	− 7	14.9	4.7	183	284	0.54	1.11
D. M.	MET	7	E	+27	+11	8.9	4.3	321	252	0.87	1.11
F. M.	I-131	22	E	+36	− 5	10.6	3.6	264	330	1.04	1.63
G. C.	I-131	10	E	+22	− 6	12.1	5.8		244	0.98	1.16
H. B.	I-131	8	E	+46	−15		6.4	130	290	0.34	1.49
K. G.	I-131	17	E				3.8		270	0.82	1.12
L. A.	I-131	10	E	+72		13.4	2.7	247	372	0.82	1.66
R. I.	I-131	7	E	+34		12.8	5.1	238	283	0.62	1.13
S. H.	$KClO_4$	11	E	+19	−20	10.5	4.4	215	241	0.94	1.12
C. T.	MET	3	T	+44	+19	9.2	8.4	198	198	0.80	0.82
D. M.	I-131	8	T	+42	+55	17.7	20.0	211	234	0.75	0.72
F. D.	I-131	9	T	+25		11.8	10.9	182		0.53	0.68
K. A.	PTU‡	4	T	+41	+29	12.0		137		0.52	1.01
M. N.	I-131	15	T	+55		9.8	11.3	169	162	0.48	0.64

* H: hypothyroid.
E: euthyroid.
T: still toxic.
† Methimazole.
‡ Propylthiouracil.

Kg. in 3 months and had a serum tocopherol concentration of 1.90 mg. per cent, and one patient with an anorexia nervosa-like syndrome whose tocopherol level was 1.21 mg. per cent notwithstanding an 18.2 Kg. weight loss over a 7-month period. Furthermore, analysis of the clinical features of the thyrotoxic group, *vide infra,* disclosed low serum tocopherols in patients who were grossly obese.

5. *Relation between clinical status and tocopherol levels among thyrotoxic patients*

Analysis of the thyrotoxic patients as to age, sex, duration of illness, extent of weight loss, clinical estimate of the severity of thyrotoxicity and prominence of muscular weakness yielded no clue upon which to base predictions of tocopherol level with complete confidence. However, from inspection of Table III it appears that, in general, patients with severe Graves' disease have the lowest levels (0.3 to 0.8 mg. per cent), while those with mild or relatively asymptomatic forms of the disease tend to values approaching normal, *e.g.,* 0.8 to 1.1 mg. per cent. Noteworthy exceptions to these generalizations are patient L. A. with very severe disease and a tocopherol concentration of 0.82, and patient R. I. who had a level of 0.62 but was essentially asymptomatic. It is clear that weight loss as such is not the critical variable. Thus, very low values of 0.52, 0.67, 0.62, and 0.52 were observed in patients F. A., P. M., R. I., and R. D., respectively, in none of whom weight loss was striking. Indeed, of these patients F. A. and P. M. were grossly obese, weighing 75.7 and 89.0 Kg., respectively. In three thyrotoxic individuals (C. E., M. E., and R. D.), muscular weakness and atrophy were dominant clinical features. All three had very low tocopherol levels, 0.54, 0.68, and 0.52 mg. per cent, respectively. One patient, M. N., with distinct shoulder girdle atrophy which had no apparent explanation other than her thyrotoxic state, was quite well preserved as to fat deposits (weight, 76.2 Kg.) and had a low serum tocopherol of 0.48 mg. per cent.

TABLE V

Response of serum tocopherol concentration to treatment of hypothyroidism

Pt.	Rx.	Duration of Rx. (*weeks*)	BMR % Pre-Rx.	BMR % Post-Rx.	PBI % Pre-Rx.	PBI % Post-Rx.	Cholesterol *mg.* % Pre-Rx.	Cholesterol *mg.* % Post-Rx.	Tocopherol *mg.* % Pre-Rx.	Tocopherol *mg.* % Post-Rx.
B. A.	T-3*	14	−21	− 2	3.4	0.4	335	251	1.92	0.83
D. A.	T-3 Ac.†	1½	−30	+ 2	1.0		339	418	2.42	1.48
G. D.	T-3	12	−26	− 4	2.7		410	184	2.43	1.01
H. D.	T-3	6	−22	+25	1.0	2.1	423	220	1.95	0.69
P. G.	D. T.‡	6	−41	−10	2.6		491	293	2.34	1.20

* T-3 = dl-triiodothyronine.
† T-3 Ac. = triiodothyroacetic acid.
‡ D.T. = desiccated thyroid.

6. Response of tocopherol concentration to change in metabolic state

In 21 of the 29 thyrotoxic patients, determinations were available after treatment with radioactive iodine (13 cases) or antithyroid drugs (8 cases). Ten of the 21 follow-up determinations were made when the patients had reached a euthyroid state, and, in 6 instances, after progression to mild to marked hypothyroidism. Of the remaining 5 patients who were still thyrotoxic, 3 were definitely improved. With the exception of the two patients who were unchanged clinically after treatment (C. T. and D. M.), there was a rise in tocopherol level in response to correction of the hyperthyroid state. As noted in Table IV, this ranged from a minimum of an 18 per cent increase in patient G. C. who had a normal level to begin with, to a 340 per cent increase 8 weeks after I^{131} treatment and return to the euthyroid state in patient H. B.

The responses of 5 hypothyroid patients to treatment with thyroid hormones are recorded in Table V and portrayed graphically in Figure 7. In each case there was a definite fall in the serum tocopherol concentration to normal levels, or, in the one case in which the patient became thyrotoxic on treatment with dl-triiodothyronine (H. D.), to subnormal levels. The acuteness of the drop in tocopherol concentration is illustrated in Figure 8, which depicts the course of one patient with classical, spontaneous myxedema during the daily intravenous administration of triiodothyroacetic acid.[2]

[2] Prepared by Dr. Rosalind Pitt-Rivers. The author appreciates the permission of Dr. Jacob Lerman to report these data.

In both thyrotoxic and hypothyroid groups, swings in tocopherol level corresponding to metabolic state were paralleled by changes in the concentration of serum cholesterol. This relationship was established in the preceding analysis in which a coefficient of correlation of + 0.801 was noted for all pairs. The imperfections in this correlation are of special interest, as in the case of the above cited patient treated with triiodothyroacetic acid, whose serum cholesterol *rose* with restoration of metabolism to normal while the tocopherol concentration *fell* (Figure 8). Conversely, in thyrotoxic patient D. M., an initial abnormally high serum cholesterol level decreased upon return to a normal metabolic state and tocopherol concentration.

DISCUSSION

These observations indicate a strong inverse relationship between levels of thyroid function and the concentration of total free tocopherols in serum. This disagrees with the stated conclusions of a recent study (33) of 16 individuals with suspected thyroid dysfunction in whom no such correlation was noted. However, low tocopherol levels were noted in 3 out of 8 patients with basal metabolic rates of + 10 or over, and in 2 of these 3 cases, increases in tocopherol concentration were observed during medical treatment of hyperthyroidism. It is likely that expansion of this series and application of discriminating tests of thyroid function would have yielded conclusions in keeping with those of the present report. The report of a high level in one hypothyroid child (34) is in accordance with the observations reported here.

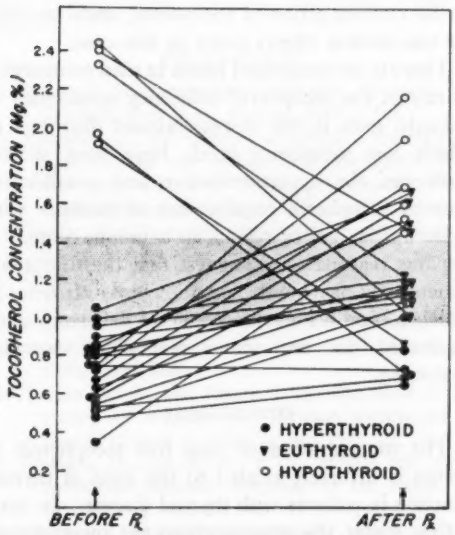

FIG. 7. GRAPHIC COMPOSITE OF CHANGES IN CONCENTRATION OF SERUM TOCOPHEROLS IN HYPO- AND HYPERTHYROID PATIENTS UNDER TREATMENT

Normal range of tocopherol concentration (2 s.d.) is indicated by stippled zone.

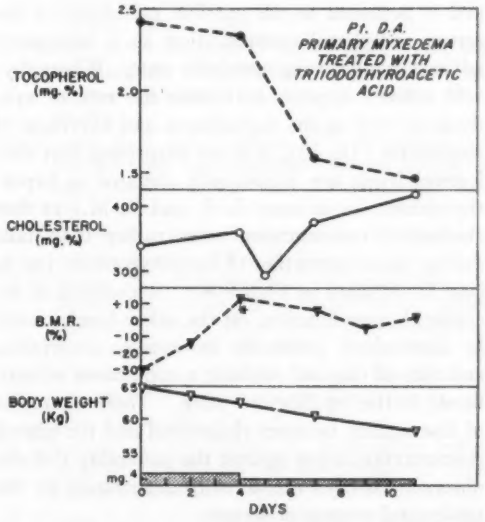

FIG. 8. THE RESPONSE OF THE SERUM TOCOPHEROL CONCENTRATION RELATED TO CHOLESTEROL LEVEL, BMR, AND WEIGHT IN A PATIENT WITH PRIMARY MYXEDEMA TREATED WITH TRIIODOTHYROACETIC ACID

The asterisk indicates technically unsatisfactory determination of the BMR on the 4th day.

The mean concentration of tocopherol in the sera of thyrotoxic patients is significantly low by normal standards set in all studies in which the method of Quaife and Harris was used (23, 35–40), as well as in relation to the internal controls. In the present series, serum levels of more than 1.2 mg. per cent rule out untreated Graves' disease, and a concentration of less than 1.7 mg. per cent is inconsistent with the diagnosis of hypothyroidism. Since low serum tocopherol levels occur in humans with absorption defects (35, 41), non-specific under-nutrition (36), and kwashiorkor (42), it is conceivable that low values observed in the thyrotoxic group relate to a deficit in absorption or diet. These are unlikely major factors in view of the isocaloric status of some patients, the lack of correlation between tocopherol level and weight, and the absence of steatorrhea and stigmata of other vitamin deficiencies. Similarly, low levels observed in some patients with liver disease (40, 43) raise the possibility that the thyrotoxic effect is mediated via deleterious influences on liver function. This mechanism cannot be excluded, but is not likely without more obvious marks of hepatic insufficiency associated with cases of liver disease in which low tocopherol levels were found.

Strong positive correlation between concentrations of total cholesterol and tocopherols in serum shows that the fat-soluble tocopherols share the reciprocal relationship of serum lipid concentrations and levels of thyroid activity (44–46). On the other hand, alteration of tocopherol concentration with the total lipid compartment or one or more of its components is obviously not critically or uniquely related to thyroid function in view of hypertocopherolemia with various hypercholesterolemic disorders (41), and the rise in serum tocopherol levels in pregnancy (39), and in patients with diabetes mellitus who have high serum cholesterol concentrations (47). This general close association of tocopherol levels with concentrations of cholesterol in serum is borne out by greater correlation between cholesterol and tocopherol levels than between specific indices of thyroid function and tocopherol levels in the overall population sampled.

Occasional dissociation of cholesterol and tocopherol concentrations is of theoretical interest,

and is pertinent to the possible usefulness of the serum tocopherol concentration as a laboratory adjunct in appraising metabolic state. Where thyroid activity appears to dictate the rate of synthesis as well as the degradation and excretion of cholesterol (18, 48), it is not surprising that cholesterol levels are occasionally *elevated* in hyperthyroidism (as in cases A. F. and D. M.) or that cholesterol concentration rises rather than falls during acute correction of hypothyroidism (as in case D. A. cited in Figure 8). Regulation of tocopherol concentration, on the other hand, would be determined primarily by intake, absorption, and rate of disposal without a component of synthesis in the equilibrated state. These examples of discrepancy between cholesterol and tocopherol concentration argue against the possibility that the observed changes are actually determined by the cholesterol content of serum.

It is impossible on the basis of serum concentration of tocopherols alone to estimate total tocopherol stores, aberrations in their metabolism, or the physiologic significance, if any, of the changes observed. It is fair, however, by analogy with low tocopherol levels in tocopherol depleted animals (49) and the fate of other metabolites in hyperthyroidism, to speculate that the low serum levels reported here reflect accelerated degradation of body tocopherol stores. Direct demonstration that this is so waits upon turnover studies or measurement of the total daily excretion of end products of tocopherol metabolism (50). Without such data one cannot exclude the possibility that the changes in tocopherol concentration reflect a direct effect of metabolic state on transport of tocopherols in serum, or an indirect one mediated by alterations in other lipids. Similarly, the physiologic impact of a depleted tocopherol supply must be appraised in terms of effects of tocopherol on the course of the thyrotoxic muscle lesion. In this connection, beneficial effects of long term therapy with oral tocopherols have been reported in 4 of 6 patients with Graves' disease (51). On the other hand, in an acute experiment with one subject, two days of treatment with tocopherols, including the phosphate ester intravenously, was accompanied by increased thyrotoxicity and weakness (52). However, the nonspecific inhibition of many enzyme systems by the phosphate (15), in a fashion seemingly unrelated to the vitamin action of tocopherol, could account for the adverse effects noted in this case.

There is no recognized lesion in man comparable to any of the tocopherol deficiency syndromes of animals, even in the aforementioned disorders in which low tocopherol levels have been shown. However, the hypermetabolism and possible increased tocopherol requirement of patients with toxic diffuse goiter results in a unique state. It remains reasonable to suggest that the thyrotoxic patient may provide the situation *par excellence* in which to find a human tocopherol deficiency state expressed as an acute reversible muscular dystrophy.

CONCLUSIONS

The concentration of total free tocopherols in serum is inversely related to the level of thyroid function in patients with thyroid disease. In toxic diffuse goiter, the concentrations are lowest among patients with severe disease, but emaciation as such is not a critical variable. Abnormalities in serum tocopherol content of thyrotoxic and hypothyroid patients generally parallel alterations in cholesterol level and are uniformly reversed upon restoration of normal metabolic state. Tocopherol concentrations are not critically correlated with clinical estimates of the extent of muscle disease in individual patients. Nonetheless, the possibility that human thyrotoxic myopathy is the analogue of vitamin E deficiency muscular dystrophy in animals merits further consideration.

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Addendum

Since submission of this manuscript, there has appeared a report of low tocopherol concentrations in the plasma of 6 hyperthyroid patients and high levels in 10 hypothyroid individuals compared with the control group of 7 normal subjects. (Costa, A., Cetini, G., Monteferrario, P., and Volterrani, O., Sulle possibili interferenze della Vitamina E con la struttura e la funzionalità tiroidea. Vitamina E: Atti del terzo Congresso Internazio-

nale Venezia 1955. Verona, Valdonega, 1956, p. 233.)

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RENAL FUNCTION IN PRIMARY ALDOSTERONISM [1]

By HARRIET P. DUSTAN, A. C. CORCORAN, AND IRVINE H. PAGE

(From the Research Division of The Cleveland Clinic Foundation and The Frank E. Bunts Educational Institute, Cleveland, O.)

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Primary aldosteronism is characterized by weakness—a sequel to potassium loss; hypertension—attributed to sodium retention; thirst and polyuria; and excretion of dilute, alkaline urine (1). These defects imply significant changes in renal function.

The purpose of this report is to describe pre- and postoperative observations of renal function in three patients suffering from aldosterone-secreting adrenal cortical tumors.

The data indicate that excess urinary potassium excretion is attributable to aldosteronism as such, and may bear on the basic cellular mechanism of action of aldosterone. The polyuria and excretion of dilute urine are recognized sequelae of potassium deficiency and probably not directly due to hormonal action. It also seems likely that renal tubular fluid as delivered from some proximal site to more distal tubules may be hypotonic rather than isotonic. The data suggest that aldosterone has profound effects on the maintenance of renal blood flow and glomerular filtration rates. From the clinical aspect, the diagnosis of aldosteronism should be strongly suspected in patients with hypertension of unknown origin who, in the presence of adequate renal excretory function, excrete alkaline urine and show loss of urinary concentrating power.

METHODS AND PROCEDURES

The three patients studied suffered from primary aldosteronism which was cured by resection of small adrenal cortical adenomata. Detailed diagnostic data together with evidences of impaired glucose tolerance are reported elsewhere (2). All three had had hypertension for years, all showed hypokalemia with hypochloremic alkalosis, impairment of urinary concentrating power and persistently alkaline urine. Preoperatively, the aldosterone content of the urine was increased in each.

Renal functions were studied pre- and postoperatively. Renal plasma flow and glomerular filtration rate were measured from the plasma clearances of para-aminohippurate (PAH) and mannitol. Total renal vascular resistance was calculated by the formula of Gomez (3). The functions of water conservation and electrolyte excretion were observed simultaneously. Water conservation was determined from the differences ($T^c_{H_2O}$) between urine flow (V) and osmolal clearance (C_{Osm}) (4) during osmotic diuresis in hydropenia (water deprivation for 16 or 24 hours and/or infusion of Pitressin®). The priming solution contained 260 mOsm. of mannitol and 0.6 gm. of PAH; the sustaining infusion supplied about 3 mOsm. of mannitol and 15 mg. per PAH per minute. Urine formed during the first 20 minutes was discarded; it was then collected from an indwelling urethral catheter at 3 to 6 successive intervals of about 10 minutes; blood was sampled prior to giving the priming solution and at intervals of about 20 minutes. Urine and plasma were analyzed for PAH and mannitol (5), chloride (6), sodium, potassium, and osmolality. Sodium and potassium determinations were done with a Baird flame photometer using lithium as the internal standard. Osmolality was determined cryoscopically, using a Fiske Osmometer.

Urinary concentrating power was measured from the nonprotein specific gravity of urine collected during the last 12 hours of a 24-hour period of fluid deprivation (5). The specimen was also used for determinations of proteinuria, pH and the urinary sediment count.

RESULTS

1. Renal hemodynamics (Table I)

(*a*) *Preoperative.* Plasma clearances of PAH (C_{PAH}) were somewhat depressed, but plasma clearances of mannitol (C_M) were within the range of normal; filtration fraction was increased in patients 1 and 3. Blood pressure was elevated in all. Renal vascular resistance (R) was increased in patients 1 and 2; this function was not calculated in patient 3. In this last patient a low C_{PAH} and very high filtration fraction indicated that renal extraction of PAH might be defective, so that C_{PAH} was not considered equivalent to renal plasma flow.

(*b*) *Postoperative.* Two weeks to two months after operation, C_{PAH} was further decreased in all, with associated decreases in C_M and decreases in filtration fraction in patients 1 and 3. Mean

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TABLE I

Hemodynamic data preoperatively and postoperatively *

Patient	Time of study	C_{PAH} ml. per min.	C_M ml. per min.	F.F.	Pm mm. Hg	R $\times 10^3$ dynes per sec. per cm^{-5}
1	Preop.	412	109	0.29	134	14.67
	Postop.					
	2 mo.	275	41	0.16	102	19.8
	6½ mo.	531	80	0.17	102	7.8
2	Preop.	443	84	0.21	166	16.83
	Postop.					
	1 mo.	367	71	0.21	157	16.49
	4 mo.	343	91	0.30	165	20.3
3	Preop.	262	113	0.48	137	
	Postop.					
	2 wk.	173	37	0.24	103	24.0
	3 mo.	218	51	0.27	123	21.9

* Patient numbers as in text. Plasma clearances (C) of p-aminohippurate (PAH) at low plasma concentrations, and of mannitol (M) in ml. per minute per 1.73 sq. m. of body surface, the data being averages of serial periods of urinary collection. Filtration fraction (F.F.), mean of averages of systolic and diastolic arterial pressures (Pm), and renal vascular resistance (R).

blood pressure (Pm) had returned to normal in patients 1 and 3 and remained high in patient 2, whereas R was abnormally high in all. Three to 6 months after operation, C_{PAH} had returned to a normal level in patient 1, was unchanged in patient 2, and was slightly increased over the last determination in patient 2. C_M had returned to normal levels in patients 1 and 2, and was somewhat increased over the previous determination in patient 3; blood pressure was at normal levels only in patient 1. R was greatly decreased since the last observation in patient 1, slightly decreased in patient 3, and somewhat increased in patient 2.

2. Water conservation (Table II)

(*a*) *Preoperative.* Although excretory functions in terms of C_{PAH} and C_M were not greatly depressed, maximal urinary nonprotein specific gravities were disproportionately low. During osmotic diuresis, either after water deprivation, during Pitressin® infusion, or in the presence of both, the urine/plasma osmolality ratio (U/P_{Osm}) approximated unity, so that water conservation function ($T^c_{H_2O}$) could be calculated only in patient 2; there was net excretion (clearance) of free water (C_{H_2O}) at rates of 0.12 and 0.71 ml. per minute in patients 1 and 3, respectively.

(*b*) *Postoperative.* Maximum urinary specific gravity was not increased in the first observations in patients 1 and 2, but had increased slightly in patient 3. In the second set of observations, there was still no change in patient 1, a slight increase in patient 2, and, as compared with the preopera-

TABLE II

Water conservation *

1 Patient	2 Time of study	3 Max. urine sp. gr. (Addis)	During osmotic diuresis 4 C_{Osm}	5 V	6 $T^c_{H_2O}$ ml. per min.	7 C_{H_2O}	8 $\frac{T^c_{H_2O}}{C_M} \times 100$	9 U/P Osm	10 Stimulus
1	Preop.	1.016	13.11	13.23		.12		.99	Hydropenia Pitressin®
	Postop.								
	2 mo.	1.015	3.96	2.44	1.52		3.77	1.62	Hydropenia
	6½ mo.	1.017	5.45	2.91	2.54		3.17	1.88	Pitressin®
2	Preop.	1.013	13.82	13.23	.59			1.04	Pitressin®
	Postop.								
	1 mo.	1.014	11.90	7.80	4.10		5.80	1.52	Hydropenia
	4 mo.	1.017	12.34	8.02	4.32		4.76	1.54	Hydropenia
3	Preop.	1.011	16.29	17.00		.71		.96	Pitressin®
	Postop.								
	2 wk.	1.016	6.40	4.82	1.58		4.20	1.33	Pitressin®
	3 mo.	1.017	10.57	8.24	2.33		4.67	1.28	Hydropenia

* Maximum urinary specific gravities during Addis concentration tests (column 3). Subsequent columns are data obtained during clearance measurements as in Table I. Osmolal clearance (C_{Osm}), urinary volume (V), maximum rate of water reabsorption in urinary concentration ($T^c_{H_2O}$), and rate of positive free water clearance (C_{H_2O}), all in ml. per minute per 1.73 sq. m. of body surface. $T^c_{H_2O}$ also is expressed in units per 100 ml. of plasma mannitol clearance. Column 9 lists the values of the ratio of urinary/plasma osmolality, and column 10 indicates the stimulus (hydropenia or Pitressin®, each alone or combined) to water conservation in the tests.

tive test, a large increase in patient 3. During osmotic diuresis, the ratio U/P_{Osm} was greater than unity, so that the water conservation function $T^c_{H_2O}$ was distinctly positive in all three patients. At the first observation, C_{Osm} was depressed in patients 1 and 3 by decreased glomerular filtration rates; calculations of $T^c_{H_2O}$ in relation to filtration rate ($T^c_{H_2O}/C_M$) indicated normal values with little change in the subsequent observations, although C_M increased in patients 1 and 3.

3. Plasma electrolytes and electrolyte excretion (*Table III*)

(*a*) *Preoperative.* Serum sodium was within the normal range; serum potassium was low and CO_2 combining power increased. In spite of the hypokalemic, hypochloremic alkalosis, urinary pH was persistently 7 or more, even during concentration tests, in which the urine is usually acid.

During osmotic diuresis, the percentage of filtered sodium which was reabsorbed varied from 95.08 (patient 1) to 91.6 (patient 3); potassium excretion (U_KV) was greatly augmented, with the ratio C_K/C_M ranging from 0.5 to 0.8. Patient 3 was given acetazoleamide (Diamox®) intravenously, with resultant further decrease in sodium reabsorption and increase in the ratio C_K/C_M from 0.79 to 1.32.

TABLE IIIA

Urine pH and plasma electrolytes

Patient	Time of study	Urine pH	Plasma electrolytes (*mEq./L.*) Na	K	Cl	CO_2
1	Preop.	7.0	145	2.8	87.5	35.8
	Postop.					
	2 mo.	5.5	145	5.6	104	17.6
	6½ mo.	5.5	145	5.3	104	22.3
2	Preop.	7.5	141	2.4	93	32.0
	Postop.					
	1 mo.	5.5	135	4.3	101	21.4
	5 mo.	5.5	148	5.0	113	19.7
3	Preop.	7.5	143	1.8	90.3	31.2
	Postop.					
	2 wk.	6.5	147	5.3	101.5	20.1
	3 mo.	5.5	145	4.7	95.9	23.1

(*b*) *Postoperative.* In each patient, hypochloremic alkalosis was replaced by acidosis, and hypokalemia by normal or slightly increased serum potassium concentrations. These changes were mild and transient in patient 3, but persistent in patients 1 and 2 at 6½ and 4 months, respectively, after operation. The urine became acid shortly after operation.

At the first observation during osmotic diuresis, the percentage of filtered sodium which was reabsorbed was decreased in patient 2 and increased in patients 1 and 3; the filtered sodium load (L_{Na}, *viz.*, $P_{Na} \times GFR$) was decreased in all.

TABLE IIIB

Electrolyte excretion *

Patient	Time of study	L_{Na} (*mEq./min.*)	$U_{Na}V$ (*mEq./min.*)	Per cent Na reab.	L_K (*mEq./min.*)	U_KV (*mEq./min.*)	C_K/C_M
1	Preop.	14.8	.727	95.08	.334	.146	.51
	Postop.						
	2 mo.	5.65	.183	96.7	.260	.053	.23
	6½ mo.	12.48	.241	98.06	.436	.043	.11
2	Preop.	13.2	.939	92.8	.296	.213	.80
	Postop.						
	1 mo.	9.6	.828	91.37	.333	.125	.37
	4 mo.	14.53	.965	93.38	.416	.114	.32
3	Preop.						
	Control	16.1	1.342	91.60	.304	.231	.78
	Diamox®	11.8	1.898	83.07	.192	.252	1.32
	Postop.						
	2 wk.	5.4	.353	93.46	.174	.062	.39
	3 mo.	7.7	.827	88.76	.234	.089	.44

* Values measured during clearance tests as in Table I. L_{Na} = plasma Na (mEq. per ml.) times C_M times 1.1 (the latter factor being used to adjust C_M to the rate of glomerular filtration); $U_{Na}V$ = urinary Na excretion; per cent Na reabsorbed was calculated as $(L_{Na} - U_{Na}V/L_{Na})100$. L_K and U_KV are, respectively, filtered load and urinary potassium excretion calculated as were the corresponding Na data. C_K/C_M expresses roughly the proportion of filtered K appearing in the urine.

TABLE IV

Proteinuria and urinary sediment counts *

Patient	Time of study	Proteinuria gm./24 hr.	Sediment counts		
			Casts	RBC millions/12 hr.	WBC
1	Preop.	0.5	0	4.0	5.0
	Postop.				
	2 mo.	0.07	0.7	1.2	0.84
	7½ mo.	0.08	1.8	2.0	0.5
2	Preop.	0.23	0.04	0.4	6.5
	Postop.				
	1 mo.	0.10	0	0.76	0.76
	4 mo.	0.13	0.25	1.4	3.0
3	Preop.	0.63	0.02	0	8.0
	Postop.				
	2 wk.	0.29	0.92	2.0	16.4
	3 mo.	0.25	0.12	0.52	10.6

* Urinary protein excretion (Addis-Shevky method) in grams per 24 hours, and urinary sediment counts for casts, red and white blood cells, in millions per 12 hours preoperatively and postoperatively.

At the second observation, L_{Na} had increased in all three, and the percentage of sodium reabsorbed was increased over the previous observation in patients 1 and 2 and decreased in patient 3; the ratio C_K/C_M was greatly decreased in the postoperative observations.

4. Proteinuria and urinary sediment (Table IV)

(*a*) *Preoperative.* There was slight proteinuria in each patient; cylindruria was absent or moderate; hematuria was present in patient 1 and increased numbers of white cells were present in all.

(*b*) *Postoperative.* Proteinuria diminished to normal ranges in patients 1 and 2 and to nearly normal in patient 3. Patients 1 and 3 showed large numbers of casts; hematuria diminished in patient 1 and appeared at a low rate in patient 2; white cell counts returned to normal in patients 1 and 2 and persisted at a high rate in patient 3.

DISCUSSION

1. Hemodynamics

(*a*) *Hypertension.* Desoxycorticosterone (DC) and NaCl elicit hypertension and hypertensive vascular disease in rats (7, 8). It is assumed that the natural mineralocorticoid, aldosterone, would do the same, but this has not been the case in experiments of brief duration in rats (9), although it has been observed when aldosterone administration is prolonged (10). Hypertension has been present in all reported cases of primary aldosteronism, although in patient 3 hypertension was never severe.

In patients 2 and 3 above and in the patient reported by Chalmers, Fitzgerald, James and Scarborough (11), hypertension did not remit, except transiently, after removal of the tumor, although it did in Conn's patient (1). Severe nephrosclerosis has been found in renal biopsy specimens from two patients (11, 12). Our patients 2 and 3 demonstrate functional evidence thereof. This nephrosclerosis may have contributed to persistence or recurrence of hypertension, perhaps by a mechanism similar to that of post-DCA hypertension in rats (13); this mechanism may be primarily renal, or may depend also on resetting of baroceptors (14). In either case, it must be assumed that aldosteronism was a primary cause of the hypertensive state and the subsequent nephrosclerosis. In patient 1 the long duration of the hypertension (26 years) and his positive family history might suggest that aldosteronism was a complication of essential hypertension. Paradoxically, this was the only patient whose hypertension was cured by removal of the tumor.

(*b*) *Renal blood flow and filtration rate.* Preoperative data from patients 1 and 2 are consistent with the presence of nephrosclerosis, leading to restriction of renal blood flow; the high filtration fraction of patient 2 suggested also defective renal extraction of PAH. This has been demonstrated in potassium-deficient patients (15); assuming a filtration fraction of 0.3, the true renal plasma flow of patient 3 would be about 400 ml. per minute and R of the order of 11. This resistance would be indicative of renal vasoconstriction and/or sclerosis.

Experience in the prolonged treatment of other forms of hypertensive disease indicated that renal resistance usually decreased with blood pressure in such a way that renal plasma flow and filtration rate did not change greatly, or that flow might even increase (16). Instead, resistance increased and filtration rate dropped, although in the last observations of patients 1 and 3 these tended to return towards normal. Two mechanisms come to mind: one is nephrosclerosis, and the other is a defect in the normal hemostasis of renal circu-

lation which might be imposed by aldosterone deficiency. That nephrosclerosis was not the cause is indicated by the fact that it does not often have this effect in nephrosclerotic patients when blood pressure is decreased by chronic treatment. Hence, speculatively, it may be that aldosterone is somehow concerned with the "autonomy of the renal circulation" (17) and that operation created a more or less persistent state of hypoaldosteronism. This supposition is supported by the abnormal postoperative electrolyte status, in which there is acidosis and a tendency toward hyperkalemia, and by the profound effects of aldosterone on renal plasma flow and filtration rate in adrenalectomized animals (18) as compared with 11-desoxycorticosterone.

2. Water conservation

Preoperatively, there was defective concentrating power during water deprivation, and also excretion of nearly isotonic urine during osmotic diuresis. These defects may be attributed either to structural loss or to the renal effects of potassium deficiency. Postoperatively, concentrating power was almost unchanged—suggesting that this defect was in fact partly structural—but water reabsorption during osmotic diuresis was restored to levels which, in relation to filtration rate, were approximately normal. This sequence indicates that the preoperative defect in water reabsorption during osmotic diuresis was a reversible defect imposed by potassium deficiency. There is an unexplained disparity between the pre- and postoperative maximum urinary specific gravities during concentration tests—these being nearly unchanged—and the large changes which took place in water reabsorptive function ($T^c_{H_2O}$) during osmotic diuresis.

This is another example of the dissociation between concentrating power measured after water deprivation and the U/P osmolal ratios attainable during osmotic diuresis, such as we (unpublished observations) and others (19) have observed in patients with severe renal disease. Even normal subjects can be brought to excrete hypotonic urine by the sudden imposition of osmotic diuresis (20), and these several examples suggest to us the possibility that distal tubular fluid is hypotonic rather than isotonic, taking the view that excretion of hypotonic urine during osmotic diuresis represents the pouring out as urine of a substantially unmodified tubular fluid which is being swept past the more distal sites of water reabsorption in urine concentration. Very recently we have been informed (Prof. H. Wirz, personal communication, 1956) that there is direct evidence in favor of this concept; fluid obtained by puncture from distal convoluted tubules of anesthetized rats was found to be hypotonic to plasma at times when the ureteral urine was distinctly hypertonic.

3. Acidification and aldosteronism

Preoperatively, our patients excreted neutral or somewhat alkaline urine, even during concentration tests. The same was true of Conn's patient (1) and of the patient of Chalmers and his associates (11). This, like the defect in water reabsorption, might represent renal injury with a defect of the mechanism of urinary acidification, notably the renal carbonic anhydrase system. However, both this system and the ammonia mechanism, although perhaps defective, are still active in aldosteronism since the patient of Chalmers and his associates (11) was able to excrete ammonia-rich, acid urine when given ammonium chloride, and our patient 3 excreted excesses of both sodium and potassium when given acetazoleamide.

The problem lies in what mechanism it may be that will cause excretion of an alkaline, potassium-rich urine in the presence of hypokalemic alkalosis. Normally, the kidney excretes an acid urine in potassium depletion despite an extracellular alkalosis, whereas the urine of our alkalotic, hypokalemic patients was normally neutral or alkaline. It can be assumed that the determinant of urine pH, of excretion of sodium and potassium, and of the ratio of basic to hydrogen ion in urine is the intracellular ionic concentration of potassium, or, as a functional reciprocal thereof, CO_2 tension (21, 22). Aldosteronism would seem to violate this assumption, since the urine is alkaline while the cell is potassium deficient and the CO_2 tension presumably increased. This paradox can be resolved if it is further assumed that intracellular potassium occurs in both bound (K) and ionized (K^+) forms (23) and that the primary mechanism of the action of aldosterone is to shift the equi-

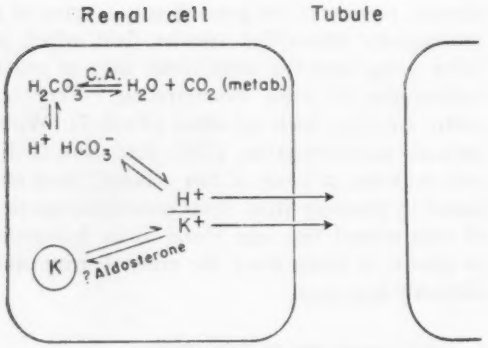

FIGURE 1

Schematic renal tubular cell indicating the relationship of hydrogen (H^+) and potassium (K^+) ions and the possibility that aldosterone may act by displacing the equilibrium between ionic (K^+) and bound (K in circle) potassium in the direction of increased ionization and excretion. C. A. denotes carbonic anhydrase.

librium $K \rightleftarrows K^+$ in the direction of increased ionization. Such increased ionization of potassium would account for the concurrence of cellular potassium depletion with formation of potassium-rich alkaline urine (Figure 1). That the phenomenon is an expression of hormonal action and not of nonspecific renal damage is indicated by the prompt recovery of this function after operative removal of the tumors.

While aldosterone causes sodium retention as well as potassium excretion, our patients showed large losses of both sodium and potassium during osmotic diuresis. Postoperatively, the excess potassium excretion (ratio C_K/C_M) promptly diminished. In contrast, the preoperative relative sodium reabsorptions of these patients were not excessive, but, in our experience, low, and the postoperative changes were irregular. We have no explanation for this finding, except as we have observed similar large defects in sodium reabsorption during osmotic diuresis in patients with severe structural renal damage due to hypertensive disease. Conceivably, the sodium-retaining action of aldosterone might be observed even during osmotic diuresis in patients whose aldosteronism had not progressed, as in our cases, into a state of advanced potassium depletion and consequent renal tubular insufficiency, complicated also by nephrosclerosis. That the latter type of damage was present preoperatively and only slowly remitted postoperatively is indicated by the functional data (Tables II, III) and by the sediment counts (Table IV).

SUMMARY AND CONCLUSIONS

Preoperatively, 3 patients with primary aldosteronism showed decreased rates of renal plasma flow, but normal rates of glomerular filtration with increased renal vascular resistance in the second patient, and probably also in the third patient. Maximal nonprotein urinary specific gravity was disproportionately low in relation to other excretory renal functions, and, during mannitol osmotic diuresis, the osmolal U/P ratios approximated unity with small net free water clearance in 2 patients. Serum potassium was low and CO_2 combining power increased, but the urinary pH was consistently 7 or more. During osmotic diuresis, the patients excreted large amounts of sodium, and also showed high rates of relative potassium clearance. One patient responded to intravenous acetazoleamide by further decreasing sodium reabsorption and increasing potassium excretion. The urine contained excess protein and white cells.

Postoperatively, the renal plasma flow and filtration rate decreased in 2 of the 3 patients and only slowly recovered. Maximal specific gravity remained low, with some tendency to recover, but the capacity to reabsorb water during osmotic diuresis was promptly restored. The patients developed acidosis and normal or somewhat increased serum potassium concentrations and the urine became acid. The defect in sodium reabsorption persisted, but the rates of potassium clearance were greatly decreased. Proteinuria and pyuria subsided completely in 2 of the 3 patients.

The excess urinary excretion of potassium seems attributable to the direct action of aldosterone on renal tubular cells and may reflect the basic mechanism of this hormone's action. The polyuria and excretion of dilute urine are attributable to tubular potassium depletion and are only indirect effects of the hormone's actions. The observations during osmotic diuresis suggest that the fluid delivered from some more proximal site (distal convoluted tubules?) to a more distal region (collecting tubules?) for concentration by water reabsorption is hypotonic rather than isotonic.

The postoperative course of renal function in association with low CO_2 combining power and a tendency to hyperkalemia suggests that aldosterone may have a function in the maintenance of renal blood flow and glomerular filtration rate.

Clinically, aldosteronism should be considered a probable diagnosis in patients with hypertension of unknown origin who, in the presence of adequate excretory renal function, excrete urine which is persistently alkaline and who show also disproportionate loss of urinary concentrating power.

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STUDIES ON THE FUNCTIONAL CAPACITY OF A DENERVATED HOMOTRANSPLANTED KIDNEY IN AN IDENTICAL TWIN WITH PARALLEL OBSERVATIONS IN THE DONOR [1]

By NEAL S. BRICKER,[2] WARREN R. GUILD,[3] JOHN B. REARDAN,[4] AND JOHN P. MERRILL [5]

(From the Department of Medicine, Peter Bent Brigham Hospital, Harvard Medical School, and the Howard Hughes Medical Institute, Boston, Mass.)

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The successful transplantation of a human kidney between 24-year-old male identical twins has made possible the study of the function of a homotransplanted kidney. The purposes of the study were twofold: 1) to evaluate the functional capacity of the transplanted kidney; 2) more specifically, to investigate the capacity of a transplanted, and therefore totally denervated, kidney to regulate renal hemodynamics and electrolyte and water excretion.

Studies were performed on both the recipient (R) and the donor (D). All but one of the experiments on R were performed after his own two kidneys had been surgically removed; thus, the majority of the data obtained specifically describes the function of the transplanted organ. Certain parallel studies were performed on D, who after transplantation possessed as his only source of renal function the mate of the transplanted kidney.

METHODS

Case material

The subjects were 24-year-old white male monozygous twins. The donor was clinically well and possessed normal renal function. The recipient was a victim of far advanced, inexorably progressive chronic glomerulonephritis of at least 16 months' duration and prior to transplantation presented the classic manifestations of uremia and malignant hypertension. After a period of preliminary study, during which time the twins were shown to be identical, D's left kidney was transplanted retroperitoneally into the right iliac fossa of R. End to end anastomoses were effected between the renal artery of the transplanted organ and the recipient's right hypogastric artery and between the renal vein and the right common iliac vein. The ureter was implanted directly into the urinary bladder.

Clinical improvement began in the immediate postoperative period and continued until all the apparent stigmata of both uremia and hypertension regressed. Bilateral nephrectomy was performed in a two-stage procedure at three and at five and one-half months following transplantation, leaving R with only the transplanted organ. To date he has enjoyed over a year of total well being on an unrestricted regimen. Detailed accounts of the clinical course and operative procedure have been previously described (1).

Procedure

Twenty-three clearance studies and several miscellaneous experiments were performed over an eighteen-month period. Control studies were obtained on D prior to transplantation. The majority of observations on R was made during a four-month interval beginning six months after transplantation.

Except where indicated, all studies were performed in the morning with the subjects postprandial and recumbent. In ten studies (six on R and four on D) glomerular filtration rate (GFR) was measured by the clearance of inulin (C_{In}) using standard renal clearance techniques as previously described (2). In nine of these ten studies, effective renal plasma flow (ERPF) was measured by the clearance of p-aminohippurate (C_{PAH}). In six of the studies in which C_{In} was measured, urine was collected through an inlying soft rubber multiholed catheter, and urine collection periods (15 to 20 minutes in duration) were concluded with two washouts of distilled water and two or more injections of air. In the control and early postoperative studies on D and the postoperative (left nephrectomy) and osmotic diuresis studies on R, bladder catheterization was not performed.

In an additional ten studies (six on R and four on D) endogenous creatinine clearance (C_{Cr}) was employed to estimate trends in GFR. These studies were carried out using voided specimens. In the majority of individual

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[2] Present address: Washington University School of Medicine, St. Louis, Missouri.

[3] Life Insurance Medical Research Fellow.

[4] U. S. Public Health Service Fellow.

[5] Established Investigator of the American Heart Association.

TABLE I *

Serial measurements of C_{in}, C_{Cr} and C_{PAH} in recipient and donor

	Time, mos. after transplant.	C_{in} ml./min.	C_{Cr} ml./min.	C_{PAH} ml./min.	F.F. %	24 hr. C_{Cr} ml./min.
Donor						
Two-kidney control		147		477	33.0	
Recipient						
Before right nephrectomy	3	67		369	18.0	
After bilateral nephrectomy	6		84			
	6	65		460	14.2	
	6		82			
	7	71		378	18.7	
	7		86			
	7	59		391	15.0	
	7		73			
	8	61		362	16.7	
	8		78			
	9		96			
	9	65	94			
	9					65
	9					68
	9					67
Donor						
After removal of left kidney	1	68		389	17.6	
	7	74		413	17.9	
	7		92			
	7		77			
	8	84		420	20.0	
	8		76			
	8		93			

* C_{in} = inulin clearance; C_{Cr} = endogenous creatinine clearance; C_{PAH} = p-aminohippurate clearance; F.F. = filtration fraction.

clearance periods, urine flows exceeded five ml. per minute, and the collection periods were generally greater than 20 minutes in duration. Finally, in three studies (on the recipient) GFR was estimated by means of 24-hour C_{Cr}. The experiments which did not include estimation of GFR will be considered in the text.

Because of the diversified nature of the experiments performed, additional comments on individual experimental protocols will be included in the discussion of results.

Inulin was determined according to the method of Roe, Epstein, and Goldstein (3); PAH according to the method of Smith, Finkelstein, Aliminosa, Crawford, and Graber (4); chloride according to the method of Van Slyke and Hiller (5); and creatinine according to the method of Bonsnes and Taussky (6). Sodium and potassium were analyzed on an internally compensated Barclay flame photometer; urine and plasma osmolalities were determined on a Fiske Osmometer; pH was determined on a Beckman pH meter; and CO_2 was determined manometrically (7).

Clearance values have not been corrected to standard body surface area. For purposes of such correction, the body surface area of the recipient was 1.71 sq.m., and of the donor 1.80 sq.m.

RESULTS

I. Serial Measurements of GFR and ERPF; Concentration and Dilution; Acidification and Alkalinization

A. Serial measurements of C_{in} and C_{PAH} (Table I)

Each datum is the mean of three clearance periods. The pretransplantation two-kidney control values in D were $C_{in} = 147$ and $C_{PAH} = 477$ ml. per minute.

Recipient: C_{in} after left nephrectomy but prior to right nephrectomy was 67 ml. per minute. Serial values for C_{in} following bilateral nephrectomy failed to reveal a consistent trend, and the average value for the five studies was 64 ml. per minute. C_{PAH} ranged from 369 to 460 ml. per minute, but again no trend was apparent. Filtration fractions varied in an irregular manner from 14.2 to 18 per cent.

C_{Cr} ranged from 73 to 96 ml. per minute with a mean of 85. The C_{Cr}/C_{in} ratio (using composite

TABLE II

Solute and water excretion during mannitol and Pitressin® infusion in the recipient *

Per.	Time min.	C_{In} ml./min.	V ml./min.	$U_{Na}V$ μEq./min.	$U_{Na}V/Fl$ %	$U_{Cl}V$ μEq./min.	$U_{K}V$ μEq./min.	Osm U/P	C_{osm} ml./min.	$Tm^{e}_{H_2O}$ ml./min.	$Tm^{e}_{H_2O}/C_{In}$ %
0	−154 to 0		0.27					2.8			
	Prime: Inulin 2.8 gm.; Pitressin® 100 mU at −47 min.; Sustain: Inulin 22 mg./min.; Pitressin® 1 mU/min. in 0.9% saline at 8.8 ml./min. from −41 to 8 min.; Inulin 22 mg./min.; Pitressin® 1 mU/min.; Mannitol 1.1 gm./min. in 0.9% saline at 8.8 ml./min. from 8 to 147 min.										
1	0 to 41	74	1.3	102	0.99	103	21	2.3	2.9	1.6	2.2
2	41 to 74	63	4.0	232	2.7	253	41	1.7	6.6	2.6	4.1
3	74 to 97	58	5.7	305	4.0	340	56	1.5	8.6	3.0	5.1
4	97 to 118	57	7.3	430	5.8	458	65	1.5	10.6	3.3	5.8
5	118 to 137	52	7.5	450	6.7	487	74	1.4	10.5	3.0	5.8
6	137 to 147	59	9.4	526	6.9	582	90	1.4	12.8	3.4	5.8

* V = urine flow; UV = minute rate of excretion; Fl = filtered load (uncorrected for Donnan equilibrium); Osm U/P = osmotic urine/plasma ratio; C_{osm} = osmolar clearance; $Tm^{e}_{H_2O}$ = maximum rate of solute free water removal. See preceding table for additional definitions.

mean values) was 1.33. C_{Cr}/C_{In} when both clearances were measured concurrently was 1.44. Values for 24-hour C_{Cr} during the administration of a 200 mg. sodium diet were 65, 68 and 67 ml. per minute, respectively.

Donor: C_{In} increased from 68 ml. per minute one week after transplantation to 84 ml. per minute eight months later. C_{PAH} increased from 389 to 420 over the same period. C_{Cr} varied in an inconsistent manner between 76 and 93 ml. per minute. C_{Cr}/C_{In} (using composite mean values for the same period of time) was 1.07.

B. Concentration and dilution (Table II)

Concentrating ability in R was assessed both by the maximal osmotic U/P ratio and by the maximal rate of solute-free water removal.[6] Following withdrawal of water for 14 hours, R achieved a maximal osmotic U/P ratio of 2.78. During osmotic diuresis induced by mannitol infusion and under the influence of Pitressin® antidiuresis (100 mU administered intravenously as a single priming dose, and 1 mU per minute by constant infusion) the maximal value for solute-free water extraction ($Tm^{e}_{H_2O}$) was 3.4 ml per minute. The ratio $Tm^{e}_{H_2O}/C_{In}$ remained constant at 5.8 per cent during the final three clearance periods.

The diluting capacity of R was judged by means of his ability to elaborate a hypotonic urine following water loading.[6] The minimal osmotic U/P ratio observed was 0.36.

The concentrating and diluting abilities of D were tested under conditions of water deprivation and water loading, respectively. The osmotic U/P ratios were of the same order of magnitude as those of the recipient.

C. Acidification and alkalinization (Table III)

The recipient was given 4.0 gm. of non-enteric coated ammonium chloride in divided doses over the course of one day. On the following morning, voided urine samples were collected under oil at approximately 30-minute intervals. After a control period, 0.25 gm. of Diamox® (acetazoleamide) was administered by mouth and five follow-up periods were obtained.

Following Diamox® administration, urinary pH rose from a control value of 5.33 to 7.63. Titratable acid disappeared within 30 minutes and bicarbonate and potassium excretion rates increased appreciably. Excretion rates of Na and Cl will be discussed below.

II. Experimental Alteration of Renal Hemodynamics

A. Intravenous Aminophylline

The influence of a single intravenous injection of 0.5 gm. of Aminophylline was examined in R

[6] The concentration process in the normal subject is characterized by two limiting values. At low rates of urine flow, solute-free water may be reabsorbed to a maximal osmotic U/P ratio of approximately four. At high rates of urine flow, such as are obtained during osmotic diuresis, the removal of solute-free water under maximal ADH stimulation is governed by an upper limit ($Tm^{e}_{H_2O}$). The normal value for $Tm^{e}_{H_2O}$ is 5.1 ± 1.5 per cent C_{In} (8). The osmotic U/P ratio in the normal subject during water diuresis does not typically decrease below 0.2.

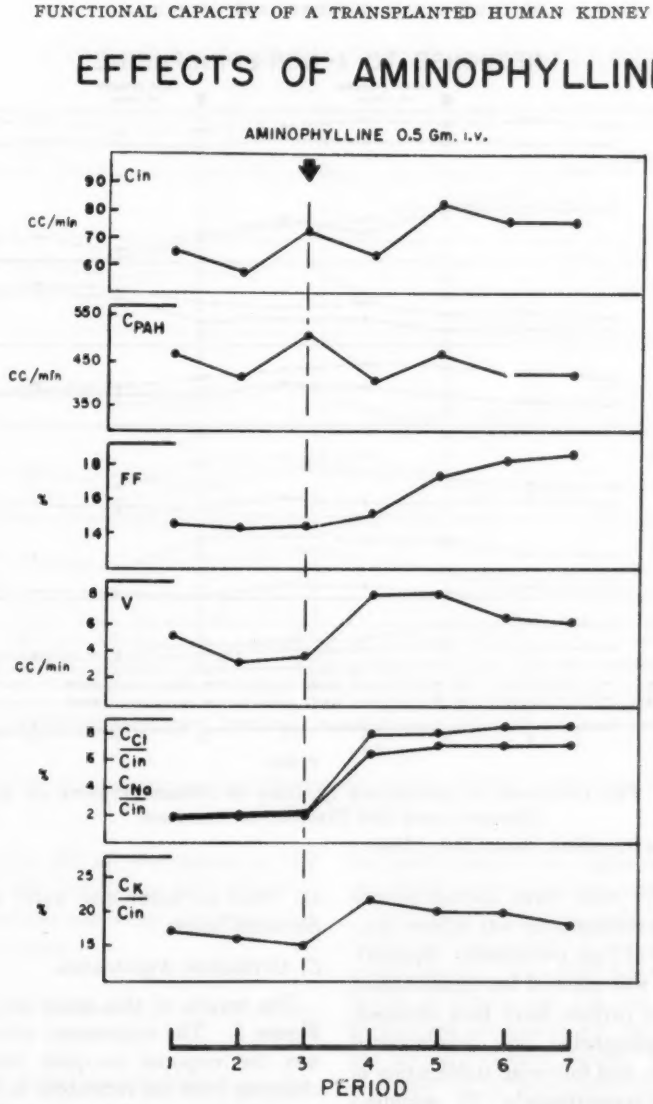

FIG. 1. THE INFLUENCE OF A SINGLE INTRAVENOUS INJECTION OF AMINOPHYLLINE ON RENAL HEMODYNAMICS, WATER AND ELECTROLYTE EXCRETION IN THE RECIPIENT

Aminophylline was injected over five minutes, and an additional five minutes was allowed before beginning the fourth period.

only (Figure 1). Subsequent to the administration of Aminophylline, C_{in} increased from a mean control value of 65 ml. per minute to a maximum of 82, whereas C_{PAH} showed a slight downward trend. Filtration fractions rose progressively from a mean control value of 14.2 to 18.4 per cent. Urine flow and electrolyte excretion values will be considered below.

B. L-norepinephrine infusion

The response of both R and D to the infusion of *l*-norepinephrine at two dosage levels was ob-

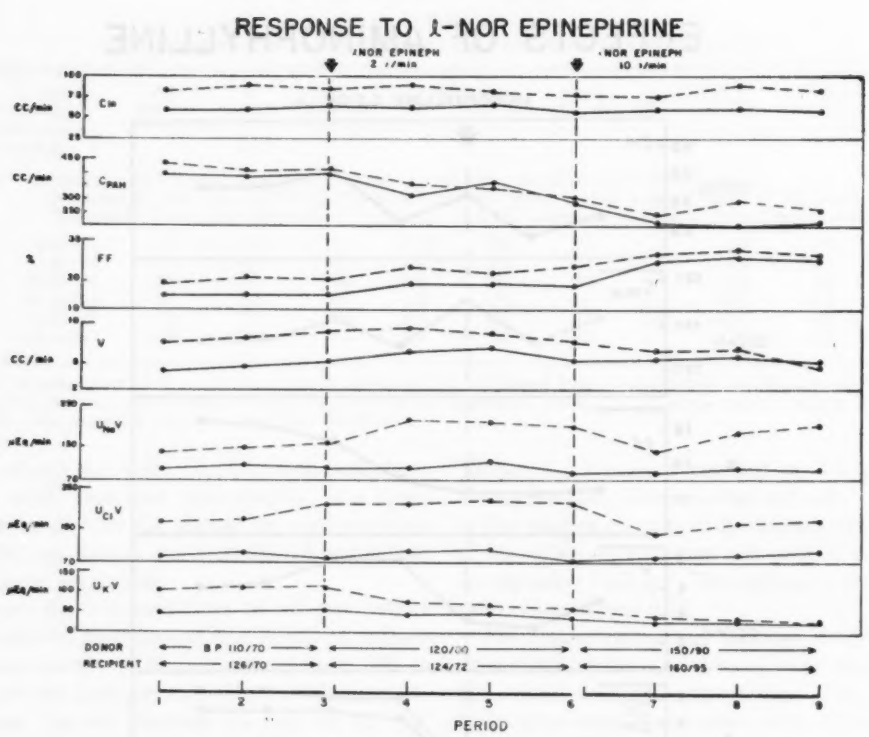

FIG. 2. THE INFLUENCE OF CONTINUOUS INFUSION OF *l*-NOREPINEPHRINE ON RENAL HEMODYNAMICS AND ELECTROLYTE EXCRETION

Solid line = recipient; dashed line = donor.

served (Figure 2). After three control periods in each subject, *l*-norepinephrine was infused continuously at the rate of 2 μg. per minute. Approximately 15 minutes was allowed for equilibration, and three clearance periods were then obtained. Thereafter, *l*-norepinephrine was administered at 10 μg. per minute, and following stabilization of blood pressures (approximately 25 minutes) three final periods were obtained.

Blood pressure did not increase appreciably in either subject during the 2 μg. dose, whereas rises of 30 to 40 mm. systolic and 20 to 25 mm. diastolic were produced by the larger dose. C_{in} remained constant throughout in both subjects. C_{PAH}, however, decreased strikingly during the infusion of the low dose, and following the increment in dosage a further decrease was demonstrable. Filtration fractions rose from a control value of 15 to 30 per cent in R and from 20 to 30 per cent in D. Data on solute and water excretion will be discussed below.

C. Orthostatic hypotension

The results of this study in R are shown in Figure 3. The experiment was intended to assess the response to quiet standing; however, changing from the recumbent to the erect position on each of two occasions was attended by symptoms and signs of peripheral vascular collapse. In both instances return to the supine position was followed by prompt improvement. It was not possible to obtain measurements of blood pressure during the periods of maximal distress, but it is probable that the actual fall exceeded that recorded in Figure 3.

During the first clearance period following hypotension, C_{in} fell from the mean control value of 61 ml. per minute to 29, and thereafter increased slowly, ultimately exceeding the control level.

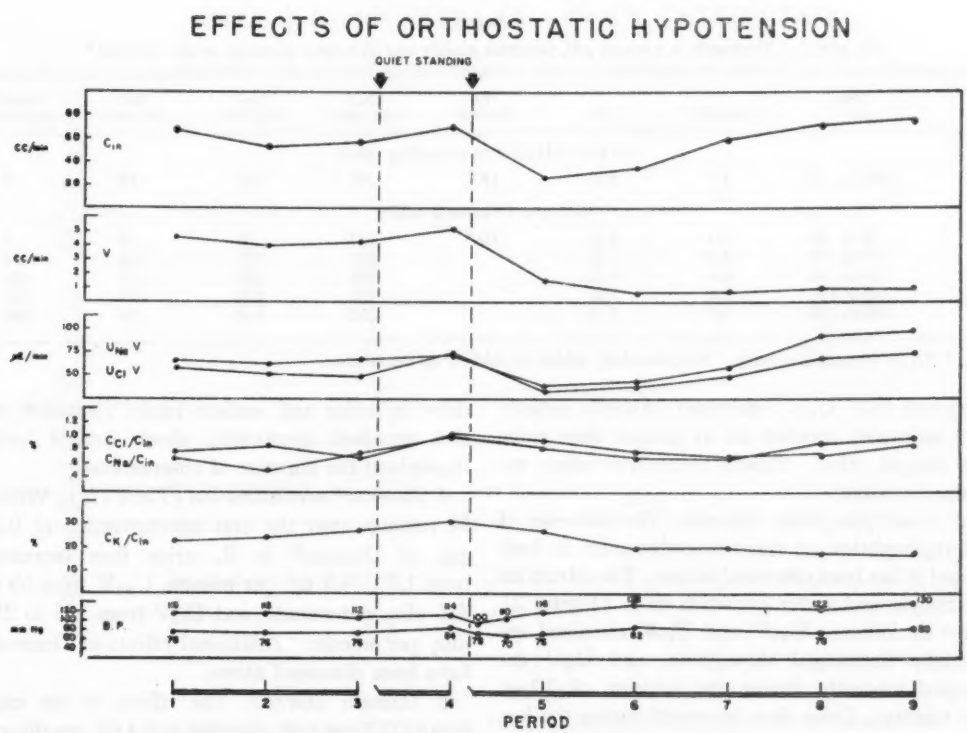

FIG. 3. THE INFLUENCE OF ORTHOSTATIC HYPOTENSION ON C_{in}, WATER AND ELECTROLYTE EXCRETION IN THE RECIPIENT

C_{PAH} decreased from 362 ml. per minute to 162 and then rose to 437 during the fifth follow-up period. The electrolyte and water excretion rates will be referred to below.

III. Experimental Alteration of Salt and Water Excretion

A. Influence of drugs presumably having an intrarenal site of action

1. Hydrocortisone: Studies were performed on R only (Table IV). He was studied in the recumbent position [7] and because of the long duration of the experiment was allowed light meals. Water diuresis was initiated by the preliminary ingestion of 750 ml. of water and maintained by the administration of supplementary oral water and intravenous 5 per cent dextrose in water.

After three control periods, 134 mg. of hydrocortisone hemisuccinate (equivalent to 100 mg. of hydrocortisone) was administered by single intravenous injection, and 134 mg. was administered by constant infusion in 500 ml. of 0.9 per cent saline over a two-hour period. Observations were continued for 11 hours after completion of the infusion.

C_{Cr} decreased moderately during the period of drug administration, but thereafter returned to the control level. Urine flow, sodium excretion rate ($U_{Na}V$), and chloride excretion rate ($U_{Cl}V$) all increased initially; however, by the ninth clearance period, a decrease in each of these values was noted. During the tenth clearance period (340 minutes after the start of the infusion) $U_{Na}V$ had decreased to a minimum value of 27.5 μEq. per minute (control 83 to 115) and urinary sodium concentration was 5.5 mEq. per L. Potassium

[7] Subjects were allowed to stand during the brief intervals of voiding to facilitate bladder emptying in this and following experiments in which catheterization was not employed.

TABLE III

The effects of Diamox® on urinary pH, titratable acidity and electrolyte excretion in the recipient *

Per.	Time min.	V ml./min.	pH	TA μEq./min.	$U_{Na}V$ μEq./min.	$U_{Cl}V$ μEq./min.	$U_{K}V$ μEq./min.	$U_{HCO_3}V$ μmol./min.
			4.0 gm. NH_4Cl day preceding study					
0	−30 to 0	1.7	5.33	18.7	99	125	40	2
			0.25 gm. Diamox® orally					
1	0 to 30	3.3	5.83	10.5	107	132	48	4
2	30 to 60	4.0	7.25		284	239	114	34
3	60 to 90	4.4	7.35		326	251	172	185
4	90 to 120	2.4	7.63		224	138	126	159
5	120 to 168	3.2	7.53		252	169	125	164

* TA = titratable acidity. See preceding tables for additional definitions.

excretion rate (U_KV) increased markedly initially and ultimately levelled off at greater than twice the control value. Plasma electrolyte values remained constant.

2. L-norepinephrine infusion: The influence of *l*-norepinephrine on renal hemodynamics in both D and R has been discussed above. The effects on electrolyte and water excretion in R (Figure 2) were as follows: $U_{Na}V$ and $U_{Cl}V$ remained essentially unchanged throughout, and U_KV decreased modestly during the infusion of 10 μg. per minute. Urine flow increased during the low dose, but returned toward control levels during the high dose.

3. Intravenous Aminophylline: The influence of 0.5 gm. of Aminophylline on renal hemodynamics in R has been discussed above. As shown in Figure 1, urine flow increased markedly, as did sodium and chloride excretion rates. The chloride/inulin and sodium/inulin clearance ratios remained appreciably above control levels throughout the duration of observations.

4. Diamox® administration (Table III): Within 90 minutes after the oral administration of 0.25 gm. of Diamox® in R, urine flow increased from 1.7 to 4.4 ml. per minute, $U_{Na}V$ from 99 to 326 μEq. per minute, and $U_{Cl}V$ from 125 to 251 μEq. per minute. Additional effects of Diamox® have been discussed above.

5. Osmotic diuresis: The effects of the infusion of 12.5 per cent mannitol at 8.8 ml. per minute in R are shown in Table II. Urine flow increased from 0.3 to 9.4 ml. per minute, and osmolar clearance (C_{osm}) from 2.9 to 12.8 ml. per minute. $U_{Na}V$ rose from 102 to 526 μEq. per minute and C_{Na}/C_{In} increased progressively from 0.99 to 6.9 per cent.

TABLE IV

The effects of intravenous hydrocortisone on C_{Cr}, solute and water excretion in the recipient *

Per.	Time min.	V ml./min.	C_{Cr} ml./min.	$U_{Na}V$ μEq./min.	$U_{Cl}V$ μEq./min.	$U_{K}V$ μEq./min.	P_{Na} mEq./L.	P_{Cl} mEq./L.	P_K mEq./L.
1	0 to 51	5.1	95	115	138	31	139		4.3
2	51 to 79	6.2	97	92	94	26			
3	79 to 112	6.5	98	83	83	25	139	108	4.3
		Hydrocortisone hemisuccinate: 134 mg. i.v. 127–139 min. and 134 mg. i.v. in 500 ml. 0.9% NaCl 139–250 min.							
4	112 to 140	6.1	85	70	61	32			
5	140 to 171	6.0	75	76	90	39			
6	171 to 230	6.8	83	107	147	70	140	108	4.3
7	230 to 290	7.6	95	105	180	114	140	108	4.4
8	290 to 350	7.5	91	74	155	115	139	108	4.4
9	350 to 410	3.7	64	33	80	65			
10	410 to 470	5.0	100	28	83	83			
11	470 to 560	4.4	99	37	88	92			
12	560 to 920	1.7	93	51	64	69			

* P = plasma. See preceding tables for additional definitions.

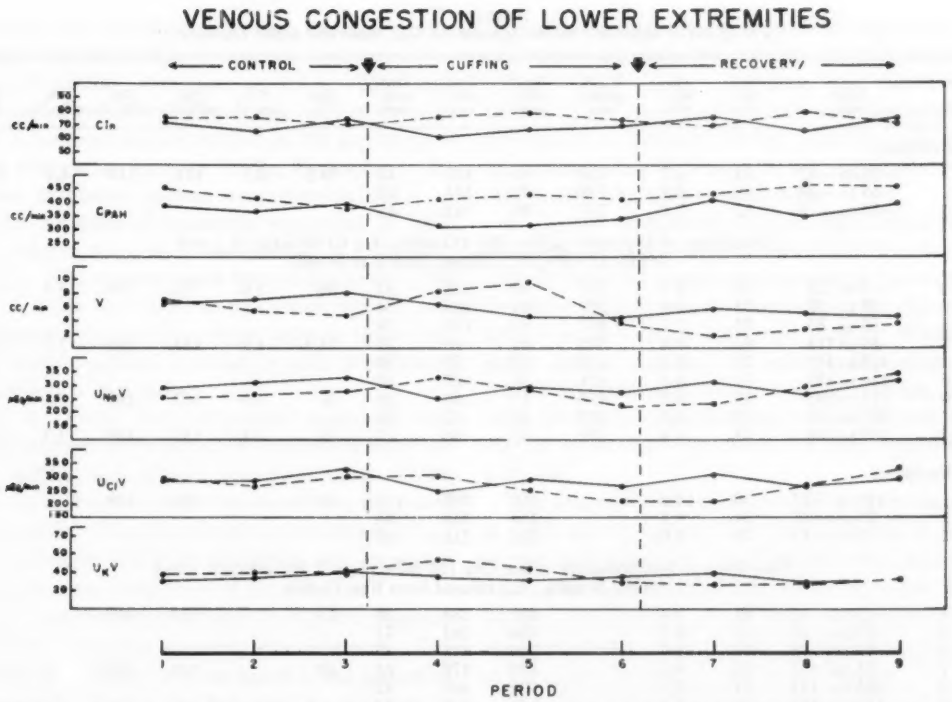

FIG. 4. THE INFLUENCE OF VENOUS CONGESTION OF LOWER EXTREMITIES ON RENAL HEMODYNAMICS AND ELECTROLYTE EXCRETION

Solid line = recipient; dashed line = donor.

B. Influence of alterations of extracellular fluid (ECF) volume and composition

1. Venous congestion of the lower extremities: The normal supine human subject responds to the inflation of blood pressure cuffs around the thighs at subdiastolic pressures by decreasing the excretion rates of sodium and water and usually decreasing glomerular filtration rate (9, 10). However, $U_{Na}V$ and $U_{Cl}V$ usually do not decrease (and often increase) during the first clearance period of cuffing (9, 10).

Experiments were performed on both R and D (Figure 4). The subjects were water loaded and maintained in positive water balance throughout. After three control clearance periods, blood pressure cuffs were inflated to just below diastolic pressures and were so maintained for approximately an hour.

Inflation of the blood pressure cuffs in R was attended by a fall in blood pressure and a concomitant decrease in C_{in}. By the third period of cuffing, blood pressure and filtration rate returned to control levels. An appreciable decrease in $U_{Na}V$ occurred only during the first period of cuffing, and the mean value for the three periods of cuffing was only 11 per cent below the mean control value. During the follow-up periods $U_{Na}V$ approximated control values. Chloride excretion rates paralleled those of sodium. A decrease in urine flow occurred, which was out of proportion to that of $U_{Na}V$ both in magnitude and duration. Values rose slightly after the cuffs were released. The urine flow response resembled that observed in the normal subject.

In D, C_{in} remained constant throughout. $U_{Na}V$ increased from the mean control value during the first period of cuffing, but fell during the succeeding two periods. During the follow-up periods, $U_{Na}V$ rose, eventually exceeding the control value. The decrease in $U_{Cl}V$ was greater in magnitude

TABLE V *

The effects of hypotonic saline infusion on C_{Cr}, solute and water excretion

Per.	Time min.	C_{Cr} ml./min.	V ml./min.	$U_{osm}V$ μosm./min.	$U_{Na}V$ μEq./min.	$U_{Cl}V$ μEq./min.	$U_{K}V$ μEq./min.	Hct %	T.P. gm. %	P_{Na} mEq./L.	P_{Cl} mEq./L.	P_{K} mEq./L.	Wt. Kg.
A. Recipient													
1	−95 to −65	83	6.7	720	94	147	48	39.5	6.5	144	110	4.2	59.6
2	−65 to −35	81	6.8	738	92	143	42						
3	−35 to − 5	82	7.1	717	99	142	41						
	Two liters of hypotonic saline (Na 112 mEq./L.; Cl 90 mEq./L.; and HCO_3 22 mEq./L.) infused from 0 to 84 min.												
4	−5 to 25	81	6.7	737	90	115	32	36	5.8	131	104	4.2	
5	25 to 55	78	6.9	589	81	113	34						
6	55 to 85	75	7.3	632	80	113	39						
7	85 to 115	83	6.8	595	64	99	37	34.5	4.9	133	105	3.7	62.5
8	115 to 146	74	6.4	498	52	75	29						
9	146 to 175	71	6.2	424	50	67	28						
10	175 to 205	79	6.2	473	43	68	26	34	4.8	132	105	3.4	
11	205 to 235	75	6.2	47[illegible]	43	67	26						
12	235 to 268	78	6.4	392	45	64	27	35	5.4	136	109	3.3	62.3
B. Recipient													
1	−105 to −75	90	10.0		310	300	111	44		140	108		
2	− 75 to −45	84	9.8		255	259	88						
3	− 45 to −15	79	9.0		212	213	95						
	Two liters of less hypotonic saline (Na 126 mEq./L.; Cl 102 mEq./L.; and HCO_3 24 mEq./L.) infused from 0 to 73 min.												
4	−15 to 15	81	9.0		191	188	79	43		139	102		
5	15 to 45	75	8.7		184	182	73						
6	45 to 75	95	11.7		260	245	87						
7	75 to 105	75	9.3		197	178	61	39		139	105		
8	105 to 135	77	9.3		172	167	52						
9	135 to 165	76	8.3		141	129	43						
10	165 to 195	99	11.7		193	160	61	40		138	107		
11	195 to 225	77	8.7		139	135	43						
12	225 to 255	57	9.3		173	145	40	40		136	107		
C. Donor													
1	−92 to −63	79	6.6	831	164	194	76	47		139	100	4.1	66.7
2	−63 to −33	76	8.2	780	156	172	79						
3	−33 to − 3	72	8.6	774	162	162	75						
	Two liters of hypotonic saline (Na 112 mEq./L.; Cl 90 mEq./L.; and HCO_3 22 mEq./L.) infused from 0 to 94.5 min.												
4	−3 to 27.5	64	9.8	833	196	179	88	46	6.7	136	100	4.0	
5	27.5 to 57.5	76	10.9	970	240	222	104						
6	57.5 to 87	75	11.6	1,032	282	249	99						
7	87 to 117		12.6					44	5.8	135	101	3.6	70
8	117 to 147	75	11.6	1,009	313	268	80						
9	147 to 178	89	12.0	996	298	247	67						
10	178 to 208	85	11.6	974	319	240	57	42	5.8	132	98	3.8	
11	208 to 237	92	12.1	1,040	336	272	58						
12	237 to 277	100	10.7	877	294	230	39	41	5.3	131	100	3.5	69.6

* Hct = hematocrit; T.P. = total protein. See preceding tables for additional definitions.

and more sustained than that in $U_{Na}V$. Urine flow decreased markedly during the third period of cuffing, and increased slightly after the first recovery period.

2. Systemic hypotension: The effects of orthostatic hypotension on renal hemodynamics in R have been discussed above (Figure 3). Presumably, the fall in blood pressure was associated with a transient decrease in effective circulating blood volume. Sodium and chloride excretion rates decreased following the onset of hypotension. However, rather than remaining depressed (as occurs in the normal subject), they increased with C_{in}, such that the sodium/inulin and chloride/inulin clearance ratios remained essentially constant throughout. Urine flow, on the other hand, de-

creased and remained depressed throughout the duration of the study.

3. Hypotonic expansion of ECF: The rapid infusion of two liters of hypotonic sodium chloride into a hydrated human subject in the supine position evokes an increase in $U_{Na}V$ despite a decrease in plasma sodium concentration (11).

The influence of hypotonic expansion of ECF was studied twice in R and once in D (Table V, A–C). The subjects were hydrated initially (500 ml. of water over a 30 to 60-minute period) and maintained in positive water balance thereafter. The control periods were begun after brisk water diuresis was in force.

In R (Table V-A) hypotonic expansion was associated with a progressive decrease in total solute, sodium and chloride excretion rates. Urine flow showed only minor changes and C_{Cr} decreased moderately. Plasma sodium concentration (P_{Na}) fell strikingly following hydration and increased modestly after completion of the infusion.

In a separate study (Table V-B), R's response to a less hypotonic solution was observed. The results were similar to those noted in the preceding experiment, despite the fact that the decrease in P_{Na} was of small magnitude.

The donor responded to hypotonic expansion in a manner characteristic of the normal subject (Table V-C). Thus, urine flow, total solute excretion, $U_{Na}V$, and $U_{Cl}V$ all increased appreciably subsequent to initiation of the infusion. C_{Cr} approximated control levels during expansion but rose during the follow-up periods.

4. Isotonic expansion of ECF: The normal human responds to the intravenous administration of isotonic saline by a moderate increase in the excretion rates of sodium and water and by the elaboration of an isotonic or hypertonic urine. When a single dose of two liters of water is administered 8 to 12 hours in advance of the saline infusion, the latter produces a copious excretion both of sodium and of water and the urine becomes hypotonic (12, 13).

The response of both R and D to the infusion of 3,000 ml. of isotonic saline at 42 to 54 ml. per minute was observed. Each subject was studied twice, once in the hydropenic state (no water for 14 hours preceding the infusion), and again in the prehydrated state having ingested two liters of tap water ten hours before the infusion.

Recipient, hydropenic state: The results of this and the prehydration study on R are shown in

TABLE VI *

The effects of isotonic saline infusion on C_{Cr}, solute and water excretion in the recipient

Per.	Time	C_{Cr}	V	Osm U/P	C_{osm}	C_{H_2O}	$U_{osm}V$	$U_{Na}V$	$U_{Cl}V$	U_KV
				Hydropenic State						
				Three liters of isotonic NaCl (Na = 144 mEq./L.) infused from 0 to 70 min.						
1	−8 to 21	67	0.9	2.1	1.9	−1.0	522	97	118	32
2	21 to 36	77	1.8	1.5	2.6	−0.8	729	135	173	52
3	36 to 51	76	3.0	0.84	2.5	0.5	699	135	203	59
4	51 to 70	63	3.4	0.59	2.0	1.8	571	89	226	63
5	70 to 82	88	5.9	0.47	2.8	3.1	781	113	187	86
6	82 to 99	67	5.8	0.38	2.2	3.5	628	92	147	73
7	99 to 117	67	6.3	0.36	2.3	4.1	646	89	152	74
8	117 to 137	59	5.4	0.37	2.0	3.4	578	108	151	63
				Prehydrated State						
				Three liters of isotonic NaCl (Na = 143 mEq./L.) infused from 0 to 56 min.						
1	−97 to −7	92	1.2	1.9	2.3	−1.1	644	172	168	23
2	− 7 to 20	84	2.9	0.82	2.4	0.5	671	185	206	40
3	20 to 39	90	4.6	0.64	2.9	1.7	820	236	270	55
4	39 to 57	83	6.2	0.49	3.1	3.2	852	218	269	67
5	57 to 73	85	7.3	0.43	3.1	4.2	863	236	283	71
6	73 to 89	81	8.6	0.37	3.2	5.1	888	241	299	79
7	89 to 104	90	8.8	0.48	3.4	4.7	950	268	324	83
8	104 to 118	87	7.5	0.54	4.1	3.4	1,155	356	412	71

* C_{H_2O} = free water clearance. See preceding tables for additional definitions.

TABLE VII *

The effects of isotonic saline infusion on C_{Cr}, solute and water excretion in the donor

Per.	Time	C_{Cr}	V	Osm U/P	C_{osm}	C_{H_2O}	$U_{osm}V$	$U_{Na}V$	$U_{Cl}V$	$U_{K}V$
				Hydropenic State						
				Three liters of isotonic NaCl (Na = 144 mEq./L.) infused from 0 to 61 min.						
1	−101 to −7	64	0.7	2.6	1.9	−1.2	548	118	132	23
2	−7 to 24	85	0.9	2.6	2.4	−1.5	683	150	172	30
3	24 to 41	83	1.5	2.0	3.0	−1.5	859	227	249	26
4	41 to 76	88	5.4	0.92	4.8	0.5	1,346	436	475	47
5	76 to 93	90	6.4	0.76	4.9	1.5	1,370	378	438	65
6	93 to 110	84	5.8	0.80	4.7	1.2	1,321	428	486	73
7	110 to 127	43	3.8	0.85	3.2	0.5	917	298	332	50
				Prehydrated State						
				Three liters of isotonic NaCl (Na = 138 mEq./L.) infused from 0 to 57 min.						
1	−45 to −8	90	2.1	1.0	2.2	−0.1	593	92	193	39
2	− 8 to 19	94	4.1	0.61	2.5	1.6	701	131	166	47
3	19 to 41	91	6.1	0.46	2.8	3.3	797	162	197	52
4	41 to 58	91	8.4	0.40	3.4	4.9	977	263	300	54
5	58 to 73	86	7.8	0.43	3.4	4.5	952	273	318	61
6	73 to 103	98	9.2	0.44	4.1	5.2	1,136	280	380	94
7	103 to 118	94	5.0	0.49	4.3	0.7	1,205	338	409	109

* See preceding tables for definitions.

Table VI. C_{Cr} showed a slight initial increase followed by a delayed fall. Urine flow increased progressively, but this was due largely to an increase in free water clearance (C_{H_2O}). As a consequence, the urine became markedly hypotonic (osmotic U/P ratio = 0.36). Osmolar clearance (C_{osm}) and sodium excretion rates exhibited only minimal and unsustained increases.

Recipient, prehydrated state: The initial values for C_{Cr}, urine flow, and electrolyte excretion rates were all higher than those noted in the hydropenic study. C_{Cr} remained constant throughout. Urine flow exhibited a progressive increase, and in this experiment, C_{osm} contributed appreciably to this. C_{H_2O} also increased and osmotic U/P ratios reached a minimum value of 0.37. $U_{Na}V$ increased gradually but progressively throughout, reaching a peak value of 356 μEq. per minute as compared to the maximal value of 135 μEq. per minute noted during the hydropenic study.

Donor, hydropenic state: The results of this and the succeeding study on D are shown in Table VII. C_{Cr}, urine flow, C_{osm}, and C_{H_2O} all increased during the first five periods. Osmotic U/P ratios decreased to a minimum of 0.76. $U_{Na}V$ rose from 118 μEq. per minute to a maximal value of 436 during the third clearance period, and thereafter decreased.

Donor, prehydrated state: Prehydration did not appear to enhance electrolyte excretion. Thus, the increases in C_{osm} and $U_{Na}V$ were smaller than those noted in the hydropenic study. The increase in C_{H_2O} was of greater magnitude, however, and osmotic U/P ratios decreased to 0.43.

5. Infusion of hyperoncotic salt poor albumin: The intravenous administration of 25 per cent salt poor human albumin into normal supine subjects has been shown to evoke a decrease in the rates of solute and water excretion (14). When the albumin is infused rapidly (*e.g.*, 100 ml. in 15 to 20 minutes) the antidiuresis is more marked (15).

The responses of both R and D to the rapid infusion of 300 ml. of salt poor human albumin are shown in Tables VIII and IX. Water diuresis was induced by the preliminary ingestion of 1,500 ml. of water and maintained by the ingestion of water in quantities slightly exceeding the urinary volumes.

In R, no trend in C_{Cr} was apparent subsequent to the initiation of the infusion. During the infusion, urine flow and C_{H_2O} decreased slightly; however, osmotic U/P ratios showed no consistent increase. C_{osm} remained constant during and following the albumin infusion, and although $U_{Na}V$ decreased progressively during the control periods,

TABLE VIII *

The effects of rapid infusion of 25% salt poor human albumin on C_{Cr}, solute and water excretion in the recipient

Per.	Time min.	C_{Cr} ml./min.	V ml./min.	C_{osm} ml./min.	C_{H_2O} ml./min.	Osm U/P	$U_{Na}V$ μEq./min.	$U_{Cl}V$ μEq./min.	$U_{K}V$ μEq./min.	P_{Na} mEq./L.	P_{Cl} mEq./L.	P_{K} mEq./L.
	1,500 ml. of water ingested from −160 to −128 min.											
1	−176 to −128	91	2.7	2.6	0.1	0.99	178	202	53	140	105	4.0
2	−128 to − 98	90	4.9	2.5	2.4	0.57	135	167	51			
3	− 98 to − 68	80	5.8	2.2	3.6	0.39	104	133	59	137	105	4.4
4	− 68 to − 39	77	5.7	2.0	3.7	0.35	74	104	57			
5	− 39 to − 8	76	5.8	2.0	3.8	0.34	73	99	52	135	105	3.8
	300 ml. albumin infused from 0 to 75 min. (First 100 ml. infused from 0 to 23 min.)											
6	−8 to 21	76	5.5	1.9	3.6	0.34	64	83	52	128	105	3.9
7	21 to 53	88	5.2	1.9	3.3	0.37	63	61	68	131	101	3.7
8	53 to 83	82	6.3	2.2	4.1	0.34	91	69	73	136	100	3.4
9	83 to 113	79	6.1	2.0	4.1	0.33	74	57	60	135	99	3.9
10	113 to 143	81	6.6	1.9	4.7	0.29	69	64	50	136	100	3.6
11	143 to 173	78	6.2	1.9	4.3	0.30	65	68	45	134	101	3.3

* See preceding tables for definitions.

the lowest value noted subsequent to initiation of the infusion was only 10 μEq. per minute less than the last control value. $U_{Cl}V$ decreased throughout the control periods, and a moderate additional fall ensued after the onset of albumin infusion.

In D, C_{Cr} varied irregularly and no trend was apparent. Urine flow, C_{H_2O}, and C_{osm} all decreased during the albumin infusion. Urine flow and C_{H_2O} increased toward control levels during the follow-up periods; however, C_{osm} remained depressed throughout. Osmotic U/P ratios increased during the albumin infusion and decreased thereafter. Both sodium and chloride excretion rates decreased markedly during the infusion.

C. Influence of 200 mg. sodium intake (Table X)

On an *ad lib.* sodium chloride intake, R excreted 158 mEq. of sodium per 24 hours. While housed on a metabolic ward he ingested a diet containing 200 mg. of sodium for a three-day period. Urinary sodium excretion was 42 mEq. during the final 24-hour period. On the morning of the fourth day the sodium concentration of a casual urine specimen was 9 mEq. per L.

The recipient was subjected to a low sodium diet for a second time after an interval of eight months (eighteen months following transplantation). On the third day of sodium deprivation, the 24-hour sodium excretion totalled 61 mEq.

TABLE IX *

The effects of rapid infusion of 25% salt poor human albumin on C_{Cr}, solute and water excretion in the donor

Per.	Time min.	C_{Cr} ml./min.	V ml./min.	C_{osm} ml./min.	C_{H_2O} ml./min.	Osm U/P	$U_{Na}V$ μEq./min.	$U_{Cl}V$ μEq./min.	$U_{K}V$ μEq./min.	P_{Na} mEq./L.	P_{Cl} mEq./L.	P_{K} mEq./L.
	1,500 ml. of water ingested from −154 to −131 min.											
1	−152 to −122	81	2.2	3.2	−1.0	1.5	242	276	37	140	114	3.9
2	−122 to − 92	73	5.1	2.6	2.5	0.51	164	201	38			
3	− 92 to − 62	99	9.0	3.2	5.8	0.35	221	241	44	139	112	4.0
4	− 62 to − 32	91	9.1	3.0	6.1	0.33	194	209	46			
5	− 32 to − 2	88	9.2	2.9	6.3	0.31	193	147	52	139	111	4.0
	300 ml. albumin infused from 0 to 40 min. (First 100 ml. infused from 0 to 9 min.)											
6	−2 to 28	79	6.9	2.5	4.4	0.36	144	106	70	134	112	4.1
7	28 to 59	84	6.3	2.3	4.0	0.37	112	59	96	136	110	3.8
8	59 to 89	92	7.7	2.4	5.3	0.31	119	74	72	137	109	3.8
9	89 to 118	90	7.8	2.3	5.5	0.30	131	79	46	137	110	3.6
10	118 to 148	97	7.9	2.3	5.6	0.29	149	93	37	136	108	3.8
11	148 to 178	84	7.9	2.4	5.5	0.31	162	108	31	136	109	3.8
12	178 to 208	87	7.7	2.4	5.3	0.31	158	110	36	136	109	3.5

* See preceding tables for definitions.

TABLE X

*The influence of a 200 mg. sodium intake on C_{Cr} and electrolyte excretion rates in the recipient**

Day	24 hr. End C_{Cr} *ml./min.*	$U_{Na}V$ *mEq./24 hr.*	$U_{Cl}V$ *mEq./24 hr.*	$U_{K}V$ *mEq./24 hr.*	P_{Na}	P_{Cl}	P_{K}
1	65	88	79	34	144	106	4.5
2	68	96	88	37	143	105	5.0
3	67	42	44	43	144	104	4.9

* See preceding tables for definitions.

DISCUSSION

The recipient twin represents an extraordinary experimental preparation in that his sole source of renal function is a transplanted kidney. Of additional interest is the fact that during the transplantation procedure the kidney became totally and irrefutably denervated. That the transplanted organ has maintained a considerable degree of functional integrity, and indeed that it functions in many respects in a normal manner is attested to by the following observations: 1) Its filtration rate approximates that of the donor's remaining kidney. 2) It has maintained its ability to concentrate urine. 3) It is able to dilute urine. 4) It is able to acidify urine. 5) It is able to alkalinize urine. 6) It is able both to increase and to decrease its rate of sodium excretion in response to varying stimuli. 7) It has the capacity to alter acutely both its filtration rate and renal plasma flow. 8) It has been able to maintain extracellular fluid volume and composition intact over a prolonged period of time despite widely varying dietary and climatic conditions.

Stability of glomerular filtration rate and renal plasma flow

In the recipient, serial studies (beginning six months after transplantation) showed neither a progressive rise nor a fall in either GFR or ERPF. It is probable, however, that some increase in clearance values occurred earlier in his course. This possibility receives indirect support from two sources: 1) Autotransplantation of a normal dog kidney to the iliac fossa using surgical techniques identical to those employed in the present subject is frequently attended by a marked initial decrease in clearance values with slow return toward control levels (16). 2) Clinical tests (including endogenous creatinine clearances) during the initial two-month period suggested improvement in renal function (1).

Serial observations on the donor over an eight-month period following unilateral nephrectomy revealed a moderate rise in GFR and ERPF.

The sum of the values for C_{in} in recipient and donor eight months following transplantation was essentially equal to the two-kidney control value in the donor (145 vs. 147 ml. per minute). The combined value for C_{PAH} at eight months was appreciably greater than the control value (782 vs. 477). The degree to which the rise in C_{PAH} represents compensatory hypertrophy is obscured by the fact that the control value may have been inordinately low (filtration fraction 33 per cent) due to the donor's state of marked apprehension.

Renal hemodynamic regulation

The recipient's responses to experimental events which typically induce changes in GFR and/or ERPF could not be distinguished from normal. Thus, following Aminophylline administration, C_{in} increased acutely with no increase in C_{PAH}; during *l*-norepinephrine infusion C_{PAH} decreased markedly while C_{in} remained constant; and following orthostatic hypotension C_{in} and C_{PAH} both decreased sharply and remained depressed after blood pressure returned to normal. These data suggest 1) that both renal vasodilatation and renal vasoconstriction may occur in the absence of renal nerves, 2) that Aminophylline and *l*-norepinephrine may both act directly on the renal vessels, and 3) that renal vasoconstriction occurring in a denervated kidney may outlast systemic vasoconstriction. The results of these studies lend some support to the conviction that the regulation of renal hemodynamics in homeostasis is not dependent upon renal nerves.

Electrolyte excretion

Two general categories of experiments were employed to study electrolyte excretion by the transplanted kidney: 1) administration of drugs which are generally conceded to influence sodium excretion at the intrarenal level; and 2) alteration of extrarenal factors which are capable of modifying sodium excretion in the normal subject.

The results of the studies included in the first category were generally indistinguishable from the normal response. Thus, following Diamox® administration, a sodium, chloride, potassium, and bicarbonate diuresis occurred accompanied by a decrease in titratable acidity. Following mannitol infusion, a progressive increase in sodium, chloride, and potassium excretion rates was observed. Following Aminophylline, a brisk sodium and chloride diuresis occurred which persisted after the filtration rate returned to control levels. Finally, following intravenous hydrocortisone, a fall in $U_{Na}V$ and $U_{Cl}V$ occurred associated with a concurrent rise in U_KV. The infusion of *l*-norepinephrine was not attended by a decrease in sodium and chloride excretion rates. However, the consistency with which this phenomenon occurs in the normal has not yet been established (17, 18).

The recipient's responses to experiments in the second category were generally qualitatively or quantitatively abnormal. In four studies, ECF volume was expanded acutely. Following infusion of hypotonic saline on two occasions, a decrease in sodium excretion rate occurred rather than the increase observed in the normal subject. Following the infusion of isotonic saline in the hydropenic state, only a minimal and unsustained increase in sodium excretion rate occurred. Finally, following infusion of hyperoncotic albumin, only a negligible decrease in sodium excretion occurred and this could not definitely be related to the albumin infusion. During inflation of blood pressure cuffs around extremities, a stimulus which presumably results in a decrease in effective circulating blood volume, the only fall in sodium and chloride excretion rates occurred early in cuffing (when the normal frequently notes an increase). Moreover, the decrease was associated with a fall in blood pressure and C_{in}, and despite continuation of cuffing, $U_{Na}V$ and $U_{Cl}V$ returned toward control levels. Following orthostatic hypotension, with its concomitant decrease in effective circulating blood volume, sodium and chloride excretion rates decreased only during the period of decreased filtration rate, and thereafter returned toward control levels with the rise in C_{in}. In the normal subject, temporary hypotension is followed by a decrease in urinary excretion of sodium and chloride which appreciably outlasts the decrease in GFR (19).

Water excretion

Three different stimuli employed in the recipient to study water excretion independent of the electrolyte excretion evoked changes: 1) infusion of Pitressin® produced a prompt antidiuresis; 2) alteration of plasma osmolality by water loading and hydropenia produced water diuresis and antidiuresis, respectively; and 3) expansion of ECF volume without change in tonicity (isotonic expansion in the hydropenic state) produced a water diuresis, and decrease in effective circulating blood volume (cuffing and orthostatic hypotension) evoked antidiureses out of proportion to the decreases in electrolyte excretion.

Consideration of abnormal responses in recipient

The only consistent deviations from normal were seen in the electrolyte excretion rates in those experiments in which ECF volume was acutely altered. Several variables should be considered when evaluating these abnormalities. First, the kidney was subjected to total anoxia for 90 minutes during the transplantation procedure. It is therefore conceivable that the defects in electrolyte excretion were due to a tubular lesion secondary to anoxemia. Somewhat against this explanation are 1) the prompt and normal response to drugs influencing sodium excretion at the intrarenal level, and 2) the many parameters of normal renal function observed including the near-normal concentrating ability.

The second consideration is that the failure to respond to volume changes was due to defective hormonal mechanisms. A deficient release of sodium-retaining hormones could also provide an explanation for the incomplete cutback in sodium excretion following ingestion of a 200 mg. sodium

diet. In this light it should be noted that the left adrenal gland was removed at the time of left nephrectomy. Evidence against the role of a hormonal abnormality is found in recent studies which suggest that Addisonian subjects respond in a normal manner to hypotonic saline (20), hyperoncotic albumin (15), and cuffing of the lower extremities (20). In addition, 24-hour excretion rates of 17-ketosteroids and 17-hydroxysteroids in the recipient were normal both in the control state and following an eight-hour ACTH infusion. Moreover, that the defect did not represent an inability of the kidney to respond to salt-retaining steroids is indicated by the normal response to intravenous hydrocortisone. The demonstration that prehydration facilitated the excretion of an administered load of isotonic saline (although to a lesser degree than that described in the two-kidney normal subject) may indicate the presence of a humoral influence on sodium excretion.

A third explanation for the defects observed in electrolyte excretion is that they were due to an abnormality in sodium transport which was present prior to transplantation of the kidney. The studies on the donor serve to minimize this possibility. Thus, his remaining kidney responded normally to hypotonic expansion of ECF, isotonic expansion of ECF in the hydropenic state, and hyperoncotic albumin infusion. The response to subdiastolic cuffing, although of small magnitude, was qualitatively and temporally normal. Finally, although the donor's response to isotonic saline infusion in the prehydrated state was abnormal, the recipient did not manifest the same abnormality.

The final and perhaps most intriguing possibility is that the recipient did not respond normally to acute changes in volume because denervation interrupted the efferent limb of a volume control system. Numerous observations in recent years have led to the belief that an extrarenal system exists which, in the interests of preserving constancy of ECF volume, is capable of influencing the renal excretion of both water and sodium. Changes in volume presumably affect water excretion by influencing the rate of release of antidiuretic hormone. However, the mechanism by which the kidney is *notified* to change its rate of electrolyte excretion in response to volume changes remains unknown. Both humoral and neurogenic pathways have been discussed (21).

That the recipient could adequately control volume on a chronic basis is attested to by his maintenance of normal body weight and plasma electrolyte concentrations over a period of many months. Moreover, that changes in volume without simultaneous changes in tonicity were capable of evoking changes in water excretion has been discussed above. There is some suggestion, however, that acute changes in volume did not evoke the changes in electrolyte excretion rates which are seen in normal subjects.

Although the present observations do not permit definitive evaluation of the possibility that renal nerves play a part in the efferent limb of the volume control system in man, the data presented are consistent with this interpretation.

SUMMARY AND CONCLUSIONS

Studies have been performed on a subject with a homotransplanted and therefore denervated kidney. The majority of experiments were performed after the subject's own two kidneys had been surgically removed, and the data therefore describe the function of the transplanted organ. Certain parallel observations were made on the donor who possessed as his only kidney the mate of the transplanted organ. The filtration rate in the donor prior to transplantation was 147 ml. per minute. Following transplantation GFR varied in the recipient between 60 to 70 ml. per minute, whereas in the donor it rose slightly from 68 to 84. Manifestations of the functional integrity of the transplanted kidney were multiple and included the abilities to concentrate, dilute, acidify and alkalinize the urine, the capacity to respond normally to experimental conditions which typically evoke acute alterations in GFR and/or ERPF, the capacity to respond normally to a sodium retaining adrenal steroid and to different diuretic agents, and the ability to maintain ECF volume and composition intact over a prolonged period of time.

Abnormalities in sodium excretion of a qualitative and/or quantitative nature were observed following acute alterations of extracellular volume. Experiments in this category included: 1) hypotonic expansion of ECF, 2) isotonic expansion of ECF in the hydropenic state, 3) hyperoncotic al-

bumin infusion, 4) venous congestion of the lower extremities, and 5) orthostatic hypotension. Four of these experiments were repeated on the donor and the response in each instance was normal.

Changes in water excretion in the recipient occurred following intravenous Pitressin®, changes in plasma osmolality, and changes in extracellular fluid volume. As examples of the latter, a marked rise in free water clearance occurred following isotonic expansion of ECF, and antidiuresis occurred in association with venous congestion of the lower extremities.

It is concluded: 1) That a transplanted kidney in an identical twin host may function normally in most respects and may not only sustain life but may maintain normality. 2) That both renal vasoconstriction and renal vasodilatation may occur in the absence of renal nerves, and that renal nerves are probably not necessary for the regulation of renal hemodynamics. 3) That renal nerves are not necessary for the acute changes in electrolyte excretion occurring after administration of either diuretic agents (carbonic anhydrase inhibitor, xanthine derivative, or osmotically active nonelectrolytic solute) or a sodium-retaining adrenal steroid. 4) That renal nerves are not necessary for changes in water excretion following intravenous Pitressin®, changes in osmotic pressure, or changes in extracellular fluid volume. 5) That renal nerves are not necessary for the chronic regulation of extracellular fluid volume or composition.

The possibility exists that the abnormalities in sodium excretion noted in the recipient following acute alteration of ECF volume occurred because denervation interrupted the efferent limb of a volume control system.

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THE ROLE OF THE KIDNEY IN THE ETIOLOGY OF RENAL HYPERLIPEMIA [1]

By N. R. Di Luzio and C. Riley Houck †

(*From The Division of Physiology, University of Tennessee, Memphis, Tenn.*)

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Progressive and continuous increases in plasma lipides after bilateral nephrectomy or ligation of the ureters have been demonstrated in cats (1), rabbits (2), dogs (3, 4) and monkeys (4). These findings led to the hypothesis that the kidney exerts a regulatory influence on plasma lipides (3). The observation that renal venous blood from normal rabbits counteracted the hyperlipemia following nephrectomy supported this concept (5). Peripheral venous or renal blood obtained from rabbits with damaged tubules did not counteract the lipemia, and it was therefore concluded that a lipide regulating factor was produced by normal tubular cells (5). Jiménez Díaz and Castro-Mendoza (6, 7) reported that a renal extract inhibited the hyperlipemia after bilateral nephrectomy or ureteral ligation in dogs. Svanborg (2), and Dimond, Marques Santos, Mundy, and Calderwood (8) failed to confirm these observations. Since the lipide alterations which occur in the nephrotic syndrome are associated with an increased incidence of atherosclerosis (9) and renal fatty degeneration, an explanation of the cause of hyperlipemia would be of value. The development of the intermittent peritoneal dialysis technique (10) as an effective means of maintaining excretory function provided an opportunity to evaluate further the role of the kidney in the etiology of renal hyperlipemia.

METHODS

Four male and 5 female mongrel dogs, weighing 14.2 to 17.9 Kg., were bilaterally nephrectomized in a single stage operation employing ether or Nembutal® anesthesia and a lumbar approach. The postoperative care and maintenance of these animals (hereafter referred to as "maintained" dogs) by the intermittent peritoneal dialysis procedure was previously described (11). Three consecutive two-hour dialyses were performed daily for the first seven postoperative days; thereafter, two daily dialyses were carried out. A low salt diet which contained 30 per cent fat, 45 per cent carbohydrate and 18 per cent protein was employed (11), and the dogs were fed via stomach tube an amount of diet equivalent to 54 Cal. per Kg. per day. Prior to and on the 7th and 14th days following nephrectomy, blood samples were withdrawn 12 to 16 hours after lavaging and feeding. The plasma was immediately separated, placed in 95 per cent ethanol, and the lipides were isolated by extraction with alcohol-ether, and re-extracted with petroleum ether. Aliquots of this extract were analyzed for total cholesterol by the procedure of Sperry and Webb (12). Phospholipide phosphorus was determined by the King method (13). Total lipides were initially determined gravimetrically, and later by the Bragdon procedure (14). The triglyceride concentration was determined as the difference between the total lipide value and the phospholipide (lipide P $\times$ 25) plus the cholesterol concentration. Non-protein nitrogen (15) also was measured in seven of the nine dogs.

RESULTS

The mean plasma lipide and non-protein nitrogen concentrations of nine maintained, bilaterally nephrectomized dogs are presented in Table I. The plasma phospholipide and cholesterol concentrations on the 7th and 14th days after nephrectomy showed no significant alterations. The plasma phospholipide/cholesterol ratio following nephrectomy remained normal. The triglyceride and total lipide levels were elevated approximately 26 per cent and 16 per cent, respectively, on the 7th and 14th days. These values were not significantly different from the control values. No lactescence of plasma was noted in these or other maintained, nephrectomized animals previously studied (11). Statistically significant changes were absent in the plasma lipide concentrations, but the variations in lipide levels were much greater in the maintained, nephrectomized dogs than in normal dogs.

The degree of azotemia as indicated by the non-protein nitrogen level (Table I) increased approximately fourfold and sixfold on the 7th and

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† Deceased, December 10, 1955.

TABLE I

The plasma lipide fractions and non-protein nitrogen concentrations in maintained bilaterally nephrectomized dogs†*

Group	Phospholipide	Cholesterol	Neutral fat	Total lipides	Non-protein nitrogen
Control	214 ± 18	120 ± 7	216 ± 27	550 ± 22	31 ± 3
7-day	223 ± 28	144 ± 17	271 ± 37	639 ± 52	128 ± 10
14-day	241 ± 32	138 ± 15	274 ± 43	650 ± 70	172 ± 8

* All values are expressed as mg. per 100 ml. of plasma.
† Mean lipide values and standard errors are derived from 9 animals.

14th days, respectively. No statistical correlation was observed between the elevated NPN levels and the plasma lipide concentrations.

We confirmed the previous reports (1–4) of hyperlipemia following nephrectomy in animals when excretory function was not maintained. Two dogs were sham-operated and three additional dogs were bilaterally nephrectomized but not maintained by peritoneal dialysis. Since nephrotic hyperlipemia is independent of exogenously administered fats (16) and occurs in fasted animals (4), these nephrectomized animals were fasted to minimize vomiting and to prolong life. Two of the nephrectomized dogs lived for seven days; the third dog lived for six days. In unmaintained, nephrectomized dogs, the mean plasma phosphatide concentration increased from 243 to 366 mg. per cent, or a mean 55 per cent elevation. The mean plasma total lipide concentration increased a comparable 56 per cent above the initial value, *i.e.*, from 660 mg. per cent to 1,060 mg. per cent. All three dogs showed an elevation in blood lipide concentrations. In contrast to the lipide increases noted in the unmaintained, nephrectomized dogs, the sham-operated fasted dogs showed a mean decrease from 290 to 200 mg. per cent, or a 31 per cent depression in the phospholipide level and a mean reduction from 680 to 550 mg. per cent in the total lipide fraction. Hematocrit values did not change in any of the five dogs. Opalescence of plasma did not occur in the three unmaintained, nephrectomized animals, but a significant hyperlipemia was uniformly observed ($P < .01$). This lipide increment in the unmaintained, nephrectomized dogs is especially significant since the control, sham-operated dogs showed a marked decrease in plasma lipide levels.

The absence of lipide alterations in the maintained, nephrectomized dogs might have resulted from a loss of lipide in the dialysate. This possibility was investigated by studying the plasma lipides of a normal sham-operated dog in which the intermittent peritoneal dialysis technique was carried out for seven days. No decline in any of the plasma lipide fractions occurred in this animal. The dialysis fluid of a nephrectomized dog contained no cholesterol, and the plasma cholesterol concentration in another maintained, nephrectomized dog, prior to and immediately after the morning dialysis, was unaltered. These observations make it unlikely that the absence of hyperlipemia in the maintained, nephrectomized dogs results from a loss of lipide in the dialyzing fluid.

DISCUSSION

Numerous investigators have studied the effects of renal ablation on the plasma lipides. Nekludow (1) noted a progressive increase in plasma cholesterol after bilateral nephrectomy or ureteral ligation in cats. Heymann (3) noted a rise in total lipide and cholesterol in dogs after bilateral nephrectomy or mercury poisoning and suggested the existence of a tubular apparatus for regulating blood lipides. Winkler, Durlacher, Hoff, and Man (4) confirmed and expanded the observations of hyperlipemia in nephrectomized monkeys and dogs.

Svanborg listed the possible causes of renal hyperlipemia as interruptions in the (a) excretory function of the kidney, (b) internal secretory activity of the kidney, or (c) metabolic function of the kidney (2, 17). Svanborg concluded that the absence of renal metabolic activity causes the elevation in blood lipides of nephrectomized animals. Our present findings of unaltered lipide levels in maintained bilaterally nephrectomized dogs do not support this conclusion.

Maintenance of bilaterally nephrectomized dogs by the peritoneal dialysis technique is associated with normal liver function, as evaluated by various metabolic and excretory liver function tests (18). Since the liver is the main organ for regulation of plasma lipides in the dog, it is entirely possible that our findings may be credited to maintenance of normal liver function by keeping the animal essentially free of toxic material(s).

Friedman and Byers (19) demonstrated that the accumulation of bile acid in plasma is a condition sufficient in itself to initiate the hepatic mechanism responsible for hypercholesteremia. Rosenman, Friedman, and Byers (20) also suggested a possible relationship between hypercholatemia and hypercholesteremia in clinical and experimental nephrosis, as well as in bilaterally nephrectomized animals (19). When the cholate: cholesterol relationship in normal rats given cholate by intravenous injection is compared with that in nephrectomized animals, it is suggestive that the rise in cholesterol concentration noted in the latter animals may exceed that which could be accounted for by the plasma cholate elevation.

In our present study, it is possible that removal of diffusible cholate from plasma of maintained nephrectomized dogs may have aided in preventing the hyperlipemia and hypercholesteremia otherwise observed.

Bloor (21) suggested that nephrotic hyperlipemia is caused by a general metabolic disturbance. Peters and Van Slyke stated that there is "no inverse relation between the serum lipides and blood non-protein nitrogen or any other index of renal excretory function" (22). It has been demonstrated, however, that hyperlipemia occurs in ureter-ligated dogs only as renal function is affected (23). The non-protein nitrogen levels observed in our maintained dogs 7 and 14 days following nephrectomy were much lower than the non-protein nitrogen levels found in non-maintained dogs 12 to 24 hours after nephrectomy (7, 23), at which time minor changes, if any, have occurred in the plasma lipide fractions (7, 23).

In consideration of the significantly different results obtained in the unmaintained and maintained nephrectomized dogs, it is apparent that the lipide changes in unmaintained nephrectomized animals are not the result of a loss in renal hormonal or metabolic activity. Our present findings question the previously proposed concepts of renal endocrine or metabolic factors which regulate plasma lipide metabolism.

SUMMARY

In bilaterally nephrectomized dogs maintained for 14 days by intermittent peritoneal dialysis, no significant alteration occurred in the plasma phospholipide and cholesterol levels. Plasma total lipide and triglyceride concentrations increased only slightly. Renal hyperlipemia induced by bilateral nephrectomy apparently results from metabolic disturbances secondary to the loss of renal excretory function and not from a loss of renal metabolic or hormonal control.

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THE HEMATOCRIT OF THE LESSER CIRCULATION IN MAN [1, 2]

By LAWRENCE S. LILIENFIELD, RENATO D. KOVACH, PAUL A. MARKS,[3] LEE M. HERSHENSON,[4] GERALD P. RODNAN,[4] FRANKLIN G. EBAUGH, JR.,[5] AND EDWARD D. FREIS

(From the Cardiovascular Research Laboratory, Georgetown University Medical Center, Washington, D. C., the Veterans Administration Hospital, Washington, D. C., and the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland)

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A discrepancy appears to exist between total blood volume as calculated from albumin space and large vessel hematocrit, and total blood volume as calculated from red cell volume and this hematocrit (1, 2). The hematocrit as measured on arterial or venous blood indicates a ratio of red cell volume to plasma volume that is higher than the total body red cell volume to total body plasma volume ratio. Fahraeus (1) in 1921 performed experiments with blood flowing through fine glass capillaries in which he demonstrated a lower hematocrit in the fine capillaries than in the larger entrance or exit vessels. It was postulated that this phenomenon was due to laminar flow with axial streaming of red cells and consequent excess of plasma. Hence, the concept of a "small vessel or capillary hematocrit" was conceived.

Freis, Stanton and Emerson (3), using T-1824 dye and differential agglutination of red cells (Ashby technique), studied the transit times of plasma and red cells through the human forearm and found a more rapid circulation for red cells than for plasma. Lawson, Overbey, Moore and Shadle (4) studied the pulmonary circulation in dogs with T-1824 dye and P^{32} tagged red cells. These authors concluded that the hematocrit of the pulmonary capillaries was much lower than that of the peripheral blood. Although Lawson's findings were consistent with a "small vessel hematocrit" concept, differences between methods used to measure red cell transit times and plasma transit times might well have affected his results.

Analysis of the data of Freis' experiments would indicate that the differences in transit times found were of insufficient magnitude to account for a lowering of the "small vessel hematocrit" by axial streaming of red cells. In an attempt to explore this phenomenon further, an investigation was made of the hematocrit of the pulmonary circulation in man.

MATERIALS AND METHODS

Eighteen studies were performed on thirteen patients without cardiovascular or pulmonary disease. Twenty ml. of a subject's blood was incubated for one hour at room temperature in a Fenwal bottle containing 9 ml. of N.I.H. Solution B acid citrate dextrose solution and approximately 50 to 100 microcuries of $Na_2Cr^{51}O_4$ equivalent to 25 to 200 micrograms of chromium metal. The mixture was centrifuged and the supernatant plasma removed. The bottle was then refilled with normal saline solution, mixed, recentrifuged and the saline supernatant removed. This was repeated until the washing saline was free of radioactivity. Twenty microcuries of I^{131} tagged human serum albumin was then added with sufficient saline to reconstitute the suspension to 20 ml. After thorough mixing a portion of this suspension was removed for analysis. The remainder (approximately 5 ml.) was then injected rapidly with a calibrated syringe into the circulation of the subject. In eleven cases the injection was made into an antecubital vein and in seven cases into the main pulmonary artery through a cardiac catheter.

Samples of blood were collected continuously at two-second intervals for one minute from a femoral artery through a seventeen gauge thin-wall needle via a short length of rubber tubing. The blood drained into paraffined test tubes containing one drop of dried heparin solution. Following collection of the samples, blood was taken for hematocrit determinations in Wintrobe tubes (2,000 G for 60 minutes). One or two ml. samples of the re-

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[3] Present address: Department of Medicine, College of Physicians and Surgeons, Columbia University, New York.

[4] Present address: Department of Medicine, Univ sity of Pittsburgh School of Medicine, Pittsburgh, P

[5] Present address: Hitchcock Foundation, Hanover, New Hampshire.

TABLE I

Pulmonary blood flow from tagged red cells and tagged plasma albumin

Subject	Injection site	Arterial hematocrit %	Plasma flow *ml./sec.*	Red cell flow *ml./sec.*	Total blood flow: Plasma flow 100-Hct./100 *ml./sec.*	Total blood flow: Red cell flow Hct./100 *ml./sec.*	Total flow from RBC / Total flow from plasma
V. C.		35.0	53.9	30.9	82	88	1.07
W. W.		41.6	92.1	65.0	155	156	1.01
A. G.		43.0	56.2	39.6	99	92	.93
E. B.		41.4	54.8	38.5	94	93	.99
W. T.	Ante-	37.0	170.5	96.2	271	260	.96
R. B.	cubital	39.0	49.6	35.5	81	91	1.12
M. S.	vein	45.5	46.1	37.4	84	82	.98
J. M.		44.0	76.5	63.5	137	144	1.05
F. I.		38.2	57.6	35.5	93	93	1.00
J. P.		44.5	61.3	45.0	110	102	.93
P. R.		43.4	95.7	66.0	169	152	.90
V. K.		43.0	34.6	26.7	61	62	1.02
L. J.		38.2	62.4	38.4	101	101	1.00
M. S.	Pulmo-	45.5	66.9	52.9	123	116	.94
J. M.	nary	42.5	69.3	49.8	121	117	.97
F. I.	artery	38.2	50.6	32.0	82	84	1.02
J. P.		44.5	60.1	41.0	106	92	.87
P. R.		42.8	61.1	50.1	107	117	1.09
Average					115	113	.99
S.D.							±.06

maining blood were pipetted into vials and centrifuged. The supernatant plasma was removed and 0.5 ml. pipetted onto planchettes containing a few granules of dry detergent. The red cells remaining in the vials were washed three times with normal saline and the Cr^{51} activity of the erythrocytes determined in a scintillation well-type counter. I^{131} activity of the plasma aliquots was determined with an end-window Geiger-Mueller tube after drying. A portion of the original injectate was analyzed in a similar manner. Because of high activity only 0.1 ml. aliquots were used for red cell activity measurement and I^{131} activity of the supernatant was determined after dilution 1:250 times with saline. Counts of Cr^{51} were converted to counts per ml. red cells from the hematocrit of the samples.

No correction for trapped plasma was made since

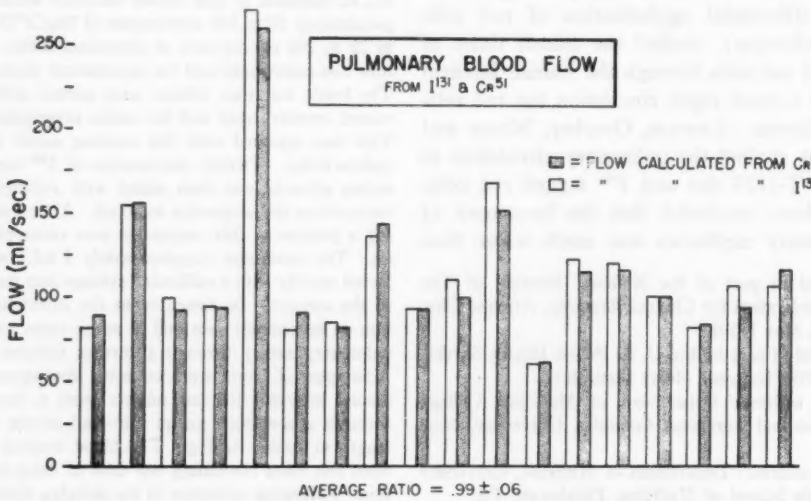

FIG. 1. COMPARISON OF PULMONARY BLOOD FLOW CALCULATED FROM LARGE VESSEL HEMATOCRIT AND EITHER TAGGED RED CELLS OR TAGGED PLASMA

TABLE II

Red cell and plasma volumes of central circulation (volume = mean circulation time × flow)

Subject	Injection site	Mean circulation time: Red cells (T_{rbc}) sec.	Mean circulation time: Plasma albumin (T_p) sec.	$\frac{T_{rbc}}{T_p}$	Red cell volume ml.	Plasma volume ml.	Central hematocrit ($Hct._c$) RBC volume / RBC vol. + Plasma vol. %	Arterial hematocrit ($Hct._a$) %	$\frac{Hct._c}{Hct._a}$
V. C.		18.8	19.1	.99	581	1,027	36.4	35.0	1.04
W. W.		13.2	13.1	1.01	857	1,204	41.5	41.6	1.00
A. G.		17.4	17.8	.98	687	1,000	40.7	43.0	.95
E. B.		22.7	23.8	.95	875	1,304	40.2	41.4	.97
W. T.	Ante-	10.2	10.9	.94	981	1,858	34.6	37.0	.94
R. B.	cubital	16.8	17.6	.95	596	837	40.6	39.0	1.04
M. S.	vein	31.6	31.7	1.00	1,182	1,461	44.7	45.5	.98
J. M.		14.7	15.0	.98	933	1,148	44.8	44.0	1.02
F. I.		25.8	26.3	.98	916	1,515	37.7	38.2	.99
J. P.		16.3	16.9	.96	734	1,036	41.5	44.5	.93
P. R.		18.4	19.1	.96	1,214	1,828	43.2	43.4	1.00
Average		18.7	19.2	.97	867	1,296			.99
S.D.				±.02					±.04
V. K.		11.6	12.4	.94	310	429	40.3	43.0	.94
L. J.		12.5	13.0	.96	480	811	35.5	38.2	.93
M. S.	Pulmo-	15.5	15.5	1.00	820	1,037	45.5	45.5	1.00
J. M.	nary	10.1	10.3	.98	502	714	45.9	42.5	1.08
F. I.	artery	13.7	14.3	.96	438	724	38.6	38.2	1.01
J. P.		11.6	12.3	.94	476	739	39.2	44.5	.88
P. R.		11.5	11.6	.99	576	708	42.2	42.8	.99
Average		12.4	12.8	.97	515	737			.98
S.D.				±.02					±.07

the hematocrits of the injectate and the samples were determined in the same manner. The Cr^{51} counts were plotted logarithmically against time, zero time being designated as the midpoint of the interval required for injection (usually two to five seconds). After extrapolation of the downslope of the resultant curve, the pulmonary red cell flow and mean circulation time were calculated by application of the Stewart-Hamilton principle (5). Similarly, the I^{131} counts were plotted and the plasma flow calculated, as well as the plasma albumin mean circulation time. The calculation of flow and mean circulation time from the curves was performed employing a method of mathematical integration to infinite time (6).

The red cell volume and plasma volume of the central circulation were computed applying the formula: volume *equals* mean circulation time *multiplied by* flow. The hematocrit of the lesser circulation was taken as the ratio of the red cell volume to the sum of the red cell volume plus plasma volume. Hematocrits also were calculated directly from the ratio of mean circulation times as discussed later.

RESULTS

Total blood flows calculated from either tagged red cells or plasma and the arterial hematocrit were almost identical (Table I, Figure 1). The ratio of these simultaneously but independently measured flows was .99 (S.D. ± .06).

The circulating red cell volumes, plasma volumes and the hematocrits of the lesser circulation are tabulated in Table II. The upper portion of the table refers to the data obtained following antecubital vein injections; the lower portion refers to the data obtained following pulmonary injection. The ratio of central circulation to large vessel hematocrit averaged .99 ± .04 (S.D.) following peripheral injection and .98 ± .07 (S.D.) following central injection. This table also indicates the mean circulation time for red cells and plasma from each injection site. In *both series* the ratio of red cell to plasma mean transit times averaged .97 ± .02 (S.D.), a figure not significantly different from unity ($P > .05$).

The total circulating central blood volumes (red cell plus plasma volumes) measured by antecubital vein injection averaged 2,163 ml. as compared to 1,252 ml. following pulmonary artery injection.

TABLE III

Red cell and plasma volumes calculated from extrapolated downslopes of plotted curves following antecubital vein injection (volume = flow per downslope)

Subject	1/Downslope: Red cells *sec.*	1/Downslope: Plasma albumin *sec.*	Red cell volume *ml.*	Plasma volume *ml.*
V. C.	10.5	10.8	324	579
W. W.	4.8	4.2	312	387
A. G.	6.4	6.5	253	365
E. B.	8.3	8.5	320	466
W. T.	3.1	3.3	298	566
R. B.	3.8	4.1	135	203
M. S.*	10.2	10.0	381	461
J. M.*	4.7	4.6	298	352
F. I.*	7.9	7.8	279	446
J. P.*	3.7	4.0	167	245
P. R.*	4.8	4.4	317	416
Average			280	408

* Subjects also had measurements made following pulmonary artery injection. See Table IV.

The large volume after antecubital vein injection can be explained by the fact that it includes the venous volume from the site of injection and all possible equitemporal injection sites.

"Pulmonary" volumes were also calculated from the extrapolated downslopes of the plotted curves according to the method described by Newman, Merrell, Genecin, Monge, Milnor and McKeever (7). The average total "pulmonary" blood volume measured 688 ml. following antecubital vein injection and 231 ml. following pulmonary artery injections (Tables III and IV). The possibility exists that this difference resulted from incomplete mixing of the indicators with pulmonary artery blood when injected through the catheter. Under this condition only a portion of the pulmonary blood volume would be measured. However, this marked difference in apparent pulmonary blood volume as measured by the Newman technique includes in the group five patients who had successive determinations made from both injection sites. In each of these five cases, the volume measured following antecubital vein injection was two and one-half to three times that calculated following pulmonary artery injection. It would seem unlikely that incomplete mixing, which should give random results, could account for the fairly constant relationship observed. Similar changes in downslope with varying injection sites, under conditions in which incomplete mixing was more probably not a factor, have been reported by Hetzel, Swan and Wood in an even larger number of patients (8). It is more likely that, under conditions of peripheral injection, the largest volume in the series through which the indicators are diluted includes the venous system and perhaps the right heart. Hence, the slope following peripheral injection would measure this volume rather than that of the lung. On the assumption that the slope following pulmonary artery injection measures pulmonary volume, the hematocrit of this "pulmonary" vasculature was calculated. The ratio of "pulmonary" to large vessel hematocrit again in these cases averaged .98 (S.D. ± .05) (Table IV, Figure 2).

TABLE IV

"Pulmonary" red cell and plasma volumes and "pulmonary" hematocrit following pulmonary artery injection (from volume = flow per downslope)

Subject	1/Downslope: Red cells *sec.*	1/Downslope: Plasma albumin *sec.*	Red cell volume *ml.*	Plasma volume *ml.*	"Pulmonary" hematocrit %	Arterial hematocrit %	Pulm. Hct. / Art. Hct.
V. K.	2.4	2.8	64	95	40.3	43.0	.94
L. J.	2.0	2.3	77	140	35.5	38.2	.93
M. S.*	2.9	2.7	151	181	45.5	45.5	1.00
J. M.*	2.0	1.8	100	118	45.9	42.5	1.08
F. I.*	2.7	2.7	86	137	38.6	38.2	1.01
J. P.*	2.4	2.3	106	138	43.4	44.5	.98
P. R.*	1.9	2.1	93	128	42.2	42.8	.98
Average	2.33	2.39	97	134			.98
S.D.							±.05

* Subjects also had measurements made following antecubital vein injection. See Table III.

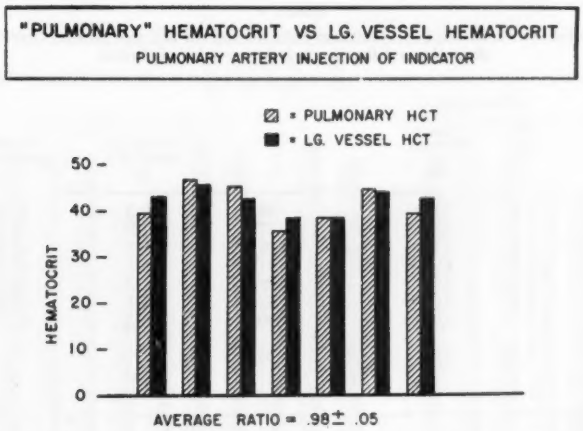

FIG. 2. COMPARISON OF HEMATOCRITS CALCULATED FROM "PULMONARY" VOLUME MEASUREMENTS (NEWMAN METHOD) WITH LARGE VESSEL HEMATOCRITS IN SEVEN SUBJECTS

DISCUSSION

These data demonstrate that during one circulation there is no measurable loss of tracer albumin by this method in the pulmonary capillaries. Had there been utilization, the total blood flow as calculated from tagged albumin would have been significantly higher than the total blood flow as calculated from the tagged red cells. Since the calculated blood flows are essentially identical the hematocrit of the lesser circulation can be recalculated directly from the ratio of mean circulation time of red cells to mean circulation time of plasma albumin as follows:

$$V_{rbc} = F_{rbc} \times T_{rbc} \qquad (1)$$

and

$$V_{pl} = F_{pl} \times T_{pl} \qquad (2)$$

where V, F, and T represent volume, flow and mean circulation time of red cells (rbc) and plasma (pl).

The central hematocrit (H_c) then is given by

$$H_c = \frac{V_{rbc}}{V_{rbc} + V_{pl}} = \frac{F_{rbc} \times T_{rbc}}{F_{rbc} \times T_{rbc} + F_{pl} \times T_{pl}} \qquad (3)$$

Since values of total pulmonary blood flow calculated on the basis of the large vessel hematocrit (H_{lv}) and the distribution of tagged red cells, or tagged plasma protein, are identical,

$$\frac{F_{rbc}}{H_{lv}} = \frac{F_{pl}}{1 - H_{lv}} \qquad (4)$$

or by rearrangement,

$$\frac{F_{rbc}}{F_{pl}} = \frac{H_{lv}}{1 - H_{lv}} \qquad (5)$$

Substituting (5) into (3), and after rearrangement, we now have

$$H_c = \frac{\frac{T_{rbc}}{T_{pl}}}{\frac{T_{rbc}}{T_{pl}} + \frac{1 - H_{lv}}{H_{lv}}} \qquad (6)$$

When the central circulation hematocrits calculated from equation six are compared to the large vessel hematocrits, the ratio again averages .98 (S.D. ± .01) following antecubital vein injection and .98 (S.D. ± .02) following pulmonary artery injection. The chief advantage of this method of calculation is that it eliminates the need for analysis of the injectate and thus reduces a possible source of laboratory error.

Similar reasoning can be applied in determining the hematocrit of the "pulmonary" circulation directly from the measured downslopes of the extrapolated curves without blood flow calculation. By this method the "pulmonary" hematocrit averages .98 ± .06 (S.D.) (Table V).

In drawing conclusions from these data it is necessary to be aware of certain limitations inherent in the method used in this study. Volumes measured by mean transit times must in-

TABLE V

"Pulmonary" hematocrit as calculated directly from extrapolated red cell and plasma time concentration curve downslopes following pulmonary artery injection

Subject	1/Downslope: Red cells (S_r) *sec.*	1/Downslope: Plasma albumin (S_p) *sec.*	$\frac{S_r}{S_p}$	Pulmonary hematocrit %	Arterial hematocrit %	$\frac{\text{Pulm. Hct.}}{\text{Art. Hct.}}$
V. K.	2.4	2.8	.86	39.3	43.0	.91
L. J.	2.0	2.3	.87	34.9	38.2	.91
M. S.	2.9	2.7	1.07	47.1	45.5	1.04
J. M.	2 0	1.8	1.11	45.1	42.5	1.06
F. I.	2.7	2.7	1.00	38.2	38.2	1.00
J. P.	2.4	2.3	1.04	45.4	44.5	1.02
P. R.	1.9	2.1	.90	40.2	42.8	.94
Average	2.33	2.39	.97			.98
S.D.						±.06

clude the blood contained in all the vessels from the injection site and all possible equitemporal injection sites to the sampling site and all possible equitemporal sampling sites. Thus, the hematocrit measured, especially following peripheral vein injection, is the average hematocrit of blood in the great vessels, heart, and the pulmonary capillaries. This amount of large vessel blood would be expected to partially mask any effect on the measured hematocrit of the blood contained in the pulmonary capillaries. Much of this large vessel blood can be eliminated from the measurement by injecting the indicators into the pulmonary artery. Though there is the possibility that mixing in the pulmonary artery is incomplete, this probably has no bearing on the hematocrit results. The albumin and red cells are well mixed before injection and should therefore have the same opportunities with respect to their pathways through the lung. Incomplete mixing with pulmonary artery blood can only result in measurement of the hematocrit of a portion, rather than the whole, of the pulmonary capillary bed. It would seem reasonable, however, that results obtained in a part of the lung would be representative of its entire capillary bed.

The average volume measured by antecubital vein injection was 2163 ml. compared to an average of 1252 ml. by pulmonary artery injection (Table II). In spite of this elimination of about 900 ml. of large vessel blood the ratios of the mean transit times for red cells and plasma remained identical ($.97 \pm .02$), as did the calculated hematocrit ratios ($.98 \pm .02$) (Figure 3). Neither of these ratios is statistically different from unity ($P > .05$), and their failure to change with injection site indicates that the circulating pulmonary hematocrit is not significantly different from the hematocrit of the larger entrance or exit vessels. The tabulated change in ratio of central hematocrit to large vessel hematocrit with injection site shown in Table II (from $.99 \pm .04$ to $.98 \pm .07$) is an artefact introduced by the statistical variations in blood flow measurement. This has been eliminated, as explained previously, in the construction of Figure 3.

These data would indicate, therefore, that in the pulmonary capillary bed of man no significant degree of axial streaming of red cells can be demonstrated by this method, and that the pulmonary capillary hematocrit, as measured by mean transit time single circulation techniques, is not significantly different from the large vessel hematocrit. Similar experiments in this laboratory utilizing the human forearm capillary beds have also failed to show evidence of significant red cell streaming in its component organs (9).

If then, as these studies suggest, red cells and plasma have the same relative volume of distribution in a single circulation in the lungs and forearm capillary beds as they do in the larger blood vessels, how can the apparent excess of plasma albumin space as measured by total body plasma volume determinations be explained? Studies of the splanchnic area in man in this laboratory (10) and independently by Lathem and Gordon (11) by an equilibration method (12), have shown a splanchnic to large vessel hematocrit ratio also of about .91. This in itself would not be sufficient

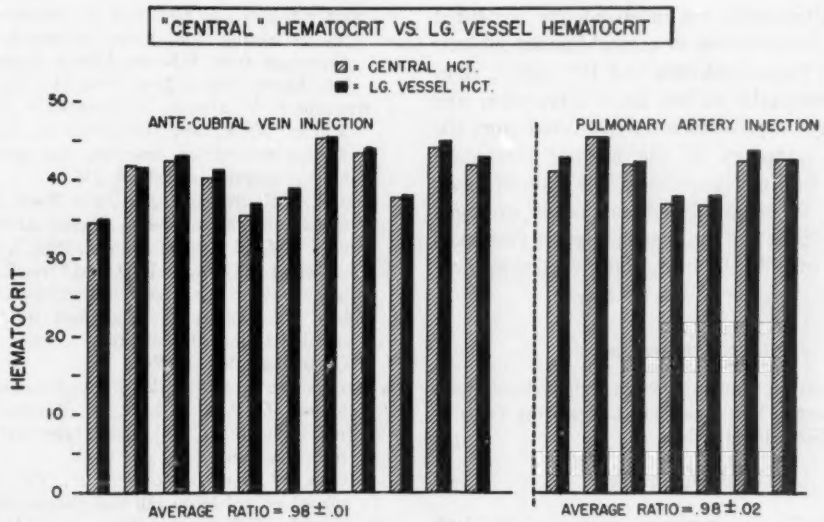

FIG. 3. CENTRAL HEMATOCRITS DETERMINED BY MEASUREMENT OF MEAN CIRCULATION TIME COMPARED WITH LARGE VESSEL HEMATOCRITS IN EIGHTEEN STUDIES

to account for the total body plasma albumin excess. The most likely explanation follows from an analysis of the methods used in the various studies. Since total body plasma measurements and splanchnic plasma volume studies require several minutes for equilibration, it seems likely that there exists a *slowly circulating* albumin space, either extravascularly or in intermittently closed capillaries containing trapped plasma, which equilibrates over several minutes with tagged albumin, and which is not measured by single circulation techniques. The observations of Rapaport, Kuida, Haynes and Dexter (13) in studying the pulmonary blood volume in dogs by both single circulation and equilibration methods would support this concept.

Another possibility to be considered is that albumin entering a capillary might not diffuse into a layer of slow moving plasma lining the capillary wall during a single circulation. The rate of mixing of entering albumin with this circumferential layer of plasma will depend upon the rate of diffusion of albumin in flowing plasma. Since albumin is of relatively large molecular size, its rate of diffusion is less than that of the smaller crystalloids and might be sufficiently small to prevent complete exchange with the circumferential layer of plasma albumin during a single circulation. Under these circumstances, the method used in this study would not be expected to measure the volume of this lining of plasma.

Failure of complete exchange of intravascular albumin does not seem likely, however, from the following consideration: if there were critical differences in mixing, due to variations in diffusion characteristics of substances of different molecular size, one would expect that small molecules would exhibit more complete exchange with the circumferential plasma layer than larger protein molecules. If this were the case the mean circulation time of albumin during a single circulation would be significantly less than that of smaller crystalloids. However, in studies of the central circulation the mean transit times of thiocyanate ion (14) and ionic Na^{24} (15) have been found to be identical with simultaneously injected labelled albumin and hence must have the same intravascular volume of distribution as the albumin. It would seem then to be valid to assume at this time that, during a single circulation, albumin mixes with all the plasma contained in a capillary into which it enters.

SUMMARY AND CONCLUSION

Red cell and plasma flows, mean circulation times, and indicator dilution curve downslopes

were simultaneously but independently measured in the lesser circulation in normal human subjects using Cr^{51} tagged red cells and I^{131} tagged albumin. Hematocrits of the lesser circulation and "pulmonary" capillaries were calculated from the data and compared to the arterial hematocrit measured in Wintrobe tubes. The ratio of lesser circulation to large vessel hematocrits averaged .98 ± .02 (S.D.). The results suggest that axial streaming of red cells in the lung capillaries does not occur to any significant extent.

ACKNOWLEDGMENTS

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THE PULMONARY BLOOD VOLUME IN MITRAL STENOSIS[1]

By ELLIOT RAPAPORT,[2] HIROSHI KUIDA,[3] FLORENCE W. HAYNES, AND LEWIS DEXTER

(From the Medical Clinic, Peter Bent Brigham Hospital, and the Department of Medicine, Harvard Medical School, Boston, Mass.)

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The Stewart-Hamilton indicator dilution method for the measurement of intrathoracic blood volume has provided the basis for an extensive study of the "pulmonary" blood volume in patients with mitral stenosis (1–7). There appears to be a lack of uniformity in the results of various studies reported in the literature, however, despite the use of the same indicator, Evans Blue dye and similar methodology. In these various studies, the only significant technical differences lay in the site of indicator injection—the most common sites being either a peripheral vein or the pulmonary artery.

Borden, Ebert, Wilson, and Wells (1), using pulmonary artery injections, found no essential difference in central blood volumes between 10 patients with mitral stenosis and 12 normal subjects. Kopelman and Lee (2) found slight, probably insignificant, increases in intrathoracic blood volume in patients with mitral stenosis as compared to normals. Most of these were studied with peripheral vein dye injections, although a small number in whom injection was made into the pulmonary artery also failed to show a significant increase. Ball, Kopelman, and Witham (3) utilized antecubital vein injections and failed to find intrathoracic blood volume greater than in normals. They reasoned that cardiomegaly in the patients with mitral stenosis results in a greater intracardiac blood volume than in the normal group and, therefore, that there must actually be a decrease of blood volume in the lung vessels. Tompkins, Burchell, and Wood (4), using peripheral injections, studied 25 patients with severe mitral disease, and 16 normals. There was a tendency for the pulmonary blood volume to be higher in the normals, although the difference was not statistically significant. Wade, Werkö, Eliasch, Gidlund, and Lagerlöf (5), using peripheral artery injections, found no difference in average volumes in 34 cases covering all the various functional subgroups of mitral stenosis. The cardio-pulmonary blood volume was seen to remain approximately a constant fraction of total blood volume. Their results confirmed previous studies from this group (6) that there is no significant change in the pulmonary blood volume in mitral stenosis, although a good correlation was shown between the cardio-pulmonary volume and the "pulmonary capillary" pressure. Finally, Doyle, Wilson, Lépine, and Warren (7) demonstrated an increased pulmonary blood volume in six patients with mitral stenosis utilizing Evans Blue dye injected through a pulmonary artery catheter.

The findings in the majority of these studies of a cardio-pulmonary blood volume not significantly different from the normal are somewhat surprising in view of the multitude of clinical, hemodynamic and pathological evidences that would tend to suggest that this volume might be increased. The present study was undertaken in an attempt to re-evaluate this problem by correlating the "pulmonary" blood volume with other variables measured by right heart catheterization; pulmonary blood volume was determined by the Bradley arteriovenous equilibration method (8, 9) as well as by the traditional Stewart-Hamilton method (10, 11, 12) and the simultaneous use of both a plasma tag, Evans Blue dye and chromium^{51} tagged red cells.

METHODS

Nineteen right heart catheterizations and pulmonary blood volume determinations were performed in 16 patients with mitral stenosis; 3 patients were studied be-

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[2] This work was done during the tenure of a Research Fellowship from the American Heart Association. Present address: V. A. Hospital, Albany, New York.

[3] This work was done during the tenure of a Postdoctorate Research Fellowship from the National Heart Institute, U. S. Public Health Service.

fore and after mitral valve surgery. Six cc. of homologous packed red cells were re-suspended in 10 cc. saline and incubated with approximately 125 microcuries of sodium chromate[51] at 37° for one hour. After centrifugation, the packed cells were washed twice with normal saline. Four cc. of the radioactive packed red cells were then pipetted into a glass beaker. An equal amount of .5 per cent Evans Blue dye solution which previously had been diluted with 1 to 2 cc. of hypertonic saline for isotonicity was added to make an 8-cc. mixture of cells and Evans Blue dye. From this mixture, 6 cc. were placed in a Gibson syringe which was calibrated by weight to deliver a known volume through the catheter. Cardiac catheterization was performed in the usual manner with a special double lumen catheter with the openings separated by 25 centimeters. After obtaining the "pulmonary capillary" pressure, the catheter was withdrawn under fluoroscopic control until the distal tip rested in the main pulmonary artery, and the proximal end opened into variable sites in the right atrium depending on the size of the heart. An 18-gauge Cournand needle was placed in each brachial artery. One minute following collection of arterial and mixed venous blood and a 3-minute sample of expired air for direct Fick cardiac output determination, the 6-cc. mixture of Evans Blue dye and chromium[51] tagged red cells was injected through the proximal lumen of the double lumen catheter into the right atrium. Injections took on the average 2 seconds. In all cases, the time of injection was plotted as the mid point of the injection interval (13). Coincident with injection, sampling was begun simultaneously from the pulmonary artery and one brachial artery by means of a constant suction pump in a manner previously described (9). Multiple samples were collected continuously at 30 to 40-second intervals for 5 or 6 periods. All samples were taken in calibrated tubes containing approximately 0.1 cc. purified dried heparin. Simultaneously, samples were collected at 2-second intervals from the Cournand needle in the opposite brachial artery in a multiple fraction collector for construction of arterial dye and red cell indicator dilution curves. Flows approximating 1.0 cc. per second were obtained through a polyethylene segment 1.0 cc. in volume. Blood samples for total plasma and red cell volume determination were collected at 10 and 20 minutes; the plasma dye concentration was extrapolated to zero time. Hematocrits were spun with a centrifuge radius arm of 19 cm. for 30 minutes at 3,000 rpm in Wintrobe tubes and corrected for trapped plasma as advocated by Gregersen (14).

One-cc. aliquots of whole blood were pipetted into culture tubes and the radioactivity determined in a well-type scintillation counter for sufficient time to insure less than a one per cent standard error. A one-cc. aliquot of the mixed red cell Evans Blue dye injectate was diluted 1 to 200 as a standard, and counted in a similar manner. The whole blood samples were then centrifuged and the removed undiluted plasma read against control plasma in a Beckman DU Spectrophotometer at 620 mu. Microcuvettes were used for determining dye concentration in the samples from which the indicator dilution curve was constructed; however, the volume of the samples obtained from the constant suction pump were sufficient to permit use of standard macrocuvettes. A dye standard was made from each patient's plasma using the hypertonic saline diluted dye injectate. The activity of the remaining packed red cells in random samples was redetermined after removal of plasma to insure that no appreciable counts had been present in the plasma.

Blood oxygen analyses were performed by the method of Van Slyke and Neill (15). Pressures were measured with Sanborn electromanometers or Statham P-23D pressure transducers and recorded on a Sanborn Poly-Viso recording oscillograph. Mitral valve area and pulmonary vascular resistance were calculated in the routine manner (16). Cardiac output was determined by both the direct Fick and by the indicator dilution method of Stewart as modified by Hamilton (10, 12). "Pulmonary" blood volume was determined as the sum of pulmonary plasma and red cell volumes by the Stewart-Hamilton method, as follows:

"Pulmonary" plasma volume

$$= \text{C.O.}^{\text{EBD}} \times \frac{\text{MTT}^{\text{EBD}}}{60} \times (1 - \text{Hct})$$

and

"Pulmonary" red cell volume

$$= \text{C.O.}^{\text{Cr}^{51}} \times \frac{\text{MTT}^{\text{Cr}^{51}}}{60} \times \text{Hct}$$

where

C.O.^{EBD} and $\text{C.O.}^{\text{Cr}^{51}}$ = Cardiac output in cc. per minute determined with Evans Blue dye and Cr^{51} tagged red cells, respectively.

MTT^{EBD} and $\text{MTT}^{\text{Cr}^{51}}$ = Mean transit time of dye or labelled red cells in seconds.

Hct = Hematocrit corrected for trapped plasma and expressed as a decimal.

Similarly, "pulmonary" blood volumes were calculated by the equilibration method of Bradley and his associates (8, 9) according to the formula:

"Pulmonary" plasma volume

$$= \frac{\text{C.O.} \times \text{T} \times (\text{PAmd} - \text{BAmd}) \times (1 - \text{Hct})}{\text{Ceq}}$$

"Pulmonary" red cell volume

$$= \frac{\text{C.O.} \times \text{T} \times (\text{PAmc} - \text{BAmc}) \times \text{Hct}}{\text{Ceq}}$$

where

C.O. = cardiac output as the average of flows determined by dye and tagged cells in cc. per second.

T = time in seconds from injection until equilibration of indicator concentration, either red cell or plasma, in the pulmonary and brachial arteries.

PAmd and PAmc = mean pulmonary arterial indicator concentration during time T in mgm. per cc. (dye) or counts per minute per cc. (cells), respectively.

BAmd and BAmc = mean brachial arterial indicator concentration during time T in mgm. per cc. (dye) or counts per minute per cc. (cells), respectively.

Ceq = indicator concentration at equilibration in mgm. per cc. (dye) or counts per minute per cc. (cells), respectively.

RESULTS

Cardiac output determined with either Cr^{51} tagged red cells or Evans Blue dye agreed well with those determined by the direct Fick method (Figure 1). The average of the ratios of cardiac outputs obtained in individual patients by the Evans Blue dye dilution method to the Fick method was .96 (s.d. = .14). The ratios of cardiac outputs obtained by the chromium^{51} tagged red cell dilution method to the outputs obtained with the direct Fick method had a mean of .96 (s.d. = .13). The average of the ratios of cardiac outputs in individual patients using Evans Blue dye to those determined with chromium^{51} red cells was 1.00 (s.d. = .05). No evidence of a systematic error could be discerned either between the indicator dilution method and the Fick method or between the two indicator dilution techniques themselves.

The mean transit time of plasma indicator was always longer than that of tagged red cells. Dye to cell mean transit time ratios obtained in individual patients averaged 1.05 (s.d. = .03).

The pulmonary hematocrit is the ratio of the pulmonary red cell volume measured by Cr^{51} to the sum of the red cell pulmonary and the Evans Blue dye plasma pulmonary volumes. When calculated by the Stewart-Hamilton method, the pulmonary hematocrit averaged 97 per cent of large vessel hematocrits (s.d. = 2 per cent) and 91 per cent (s.d. = 7 per cent) when calculated by the Bradley method. Total body hematocrit, the ratio of total red cell volume to total red cell and plasma

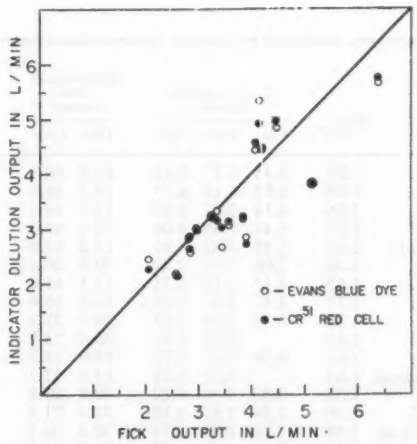

FIG. 1. THE CARDIAC OUTPUT IN MITRAL STENOSIS

Simultaneous cardiac outputs obtained by the use of chromium^{51} tagged red cells, Evans Blue dye and the direct Fick methods.

TABLE I

Pt.	Pulmonary red cell volume cc./M.² Indicator dilution	Pulmonary red cell volume cc./M.² Equilibration	Pulmonary plasma volume cc./M.² Indicator dilution	Pulmonary plasma volume cc./M.² Equilibration	Pulmonary blood volume cc./M.² Indicator dilution	Pulmonary blood volume cc./M.² Equilibration	Pulmonary hct. % Indicator dilution	Pulmonary hct. % Equilibration	Large vessel hct. %	Body hct. %	Total red cell volume cc./M.²	Total plasma volume cc./M.²	Total blood volume cc./M.²
K. K.	522	202	889	487	1,411	689	37.0	29.3	38.6	34.1	1,371	2,645	4,016
B. P.	341	243	323	351	664	594	51.4	40.9	52.4	50.7	1,047	1,018	2,065
F. D.	307	338	353	389	660	727	46.5	46.5	49.9	48.2	822	882	1,704
L. T.	661	566	657	640	1,318	1,206	50.1	46.9	49.7	45.7	1,496	1,792	3,288
M. deJ.	244	219	355	305	599	524	40.7	41.7	42.7		1,003		3,640*
A. W.	221	146	374	277	595	423	37.1	34.5	39.6	36.9	716	1,225	1,941
L. D.	248	230	368	369	616	599	40.2	38.4	41.3	41.4	855	1,207	2,062
B. G.	338	321	420	399	758	720	44.6	44.6	45.1	43.3	1,027	1,345	2,372
J. D.	342	288	445	518	787	806	43.5	35.8	44.4	42.3	1,108	1,508	2,616
K. U.	316	323	497	521	813	844	38.8	38.3	41.9	38.8	915	1,443	2,358
R. B.	414	345	572	551	986	896	42.0	38.5	43.2	41.9	1,225	1,702	2,927
Post-op	241	270	462	460	703	730	34.3	27.2	33.3	31.3	948	2,084	3,032
J. F.	275	190	424	312	699	502	38.4	37.9	40.2	37.6	738	1,225	1,963
M. C.	295		397		692		42.7		43.4			2,462	3,902*
Post-op	248		393		641		38.7		40.3	33.7	807	1,592	2,399
L. P.	322	256	354	279	676	535	47.6	47.8	48.5	43.0	982	1,302	2,284
Post-op	228	158	397	299	625	457	36.4	34.5	37.0	33.5	745	1,477	2,222
L. B.	463	266	572	339	1,035	607	44.7	44.0	46.1	41.1	1,153	1,653	2,806
A. N.	462	274	551	317	1,013	591	45.6	46.4	45.6	40.8	1,132	1,642	2,774

* Based on large vessel hematocrit.

TABLE II

Pt.	B.S.A. *M.²*	Cardiac output *L./min.*			Mean transit time *seconds*		Pressures *mm. Hg*				Pulmonary vascular resistance *dynes sec. cm.⁻⁵*	Mitral valve area *cm.²*	
		Fick	Dye	Cr^{51}	Dye	Cells	BA	"PC"	PA	RA		Gorlin formula	Surgeon's estimate
K. K.	1.59	3.35	3.33	3.16	41.5	40.8	124/74(91)	32	83/38(52)	17	470	0.6	
B. P.	1.98	4.08	4.43	4.59	18.2	16.9	137/77(95)	18	35/19(25)	9	98		
F. D.	1.88	4.14	5.32	4.93	14.9	14.1	128/92(104)	22	37/22(26)	6	77	1.1	1.2
L. T.*	1.79	3.44	2.69	3.04	52.1	47.0	117/79(94)	24	57/38(46)	10	512	0.6	2.3
M. deJ.	1.55	5.14	3.81	3.81	15.2	13.9	98/58(74)	19	31/15(23)	0	150	1.2	0.7
A. W.	1.38	2.08	2.45	2.27	20.9	20.3	100/75(84)	23	73/41(53)	5	800	0.6	0.9
L. D.	1.45	4.19	4.44	4.51	12.3	11.6	97/53(76)	15	28/13(19)	0	138	1.1	1.0
B. G.	1.77	4.47	4.85	4.98	16.8	16.0	116/75(95)	13	29/13(19)	2	142	1.1	1.0
J. D.	1.97	3.23	3.23	3.23	29.3	28.2	112/72(85)	30	68/41(53)	7	575	0.6	0.8
K. U.	1.41		3.63	3.41	20.0	18.7	98/61(80)	11	26/9(16)	−1	90	0.9	0.9
R. B.	1.87	6.38	5.67	5.75	19.9	18.7	94/60(79)	17	42/21(29)	4	118	1.0	1.0
Post-op	1.84		5.61	6.07	13.6	13.2	108/65(78)	12	23/12(17)	8	85	1.6	
J. F.	1.52	3.83	3.20	3.22	20.2	19.4	120/67(83)	29	72/32(43)	4	292	0.8	0.4
M. C.	1.50	2.84	2.82	2.88	22.4	21.3	113/69(83)	27	104/41(65)	9	914	0.6	0.8
Post-op	1.49	3.90	2.87	2.74	20.5	20.1	116/68(87)	21	100/40(63)	11	841	0.9	
L. P.	1.63	2.83	2.59	2.67	26.0	24.3	98/73(87)	22	42/23(30)	6	312	0.5	
Post-op	1.63	3.59	3.09	3.14	19.9	19.1	103/69(81)	13	40/12(21)	8	214	1.4	
L. B.	1.58	2.98	3.01	2.99	33.4	31.8	147/76(105)	20	50/19(28)	12	242	0.5	
A. N.	1.33	2.51	2.19	2.15	37.6	36.9	162/84(116)	22	40/23(30)	15	207	0.6	

* Predominant mitral insufficiency at operation.

volume, averaged 92 per cent (s.d. = 4 per cent) of large vessel hematocrits.

Table I lists the values calculated for the pulmonary blood volume by the two methods. The circulating pulmonary blood volume determined by the Stewart-Hamilton method averaged 805 cc. per $M.^2$ (range of 595 to 1,411) representing the sum of mean pulmonary plasma and red cell volumes of 463 cc. per $M.^2$ and 341 cc. per $M.^2$, respectively. The pulmonary blood volume averaged 31.9 per cent of total blood volume (range 23.2 to 40.1 per cent).

The circulating pulmonary blood volume determined as the sum of the plasma and red cell volumes by the arteriovenous equilibration method averaged 674 cc. per $M.^2$ (range of 423 to 1,206) representing the sum of an average pulmonary plasma volume of 401 cc. per $M.^2$ and red cell volume of 273 cc. per $M.^2$ The pulmonary blood volume by this method averaged 27.2 per cent of total blood volume (range 17.1 per cent to 42.7 per cent).

Table II lists the pertinent hemodynamic data obtained at catheterization in these patients.

DISCUSSION

Cardiac output

Cardiac output can be accurately calculated by the indicator dilution technique with use of only a single indicator, either red cell or plasma, since the presence of a significantly lower pulmonary hematocrit compared to large vessel hematocrit is of no consequence in the determination of cardiac output by these methods (13). Figure 1 illustrates the correlation between cardiac outputs obtained by the $chromium^{51}$, dye and Fick methods. These results lend further confirmation to the generally accepted view that arterial indicator dilution curve values of cardiac output are closely comparable to those obtained by the direct Fick method. The observations of Eliasch (17) that grades III and IV patients with mitral stenosis had average dye outputs significantly higher than Fick outputs were not confirmed in this study.

The mean of the ratios of individual Evans Blue dye to $chromium^{51}$ red cell outputs was 1.00 with only a 5 per cent standard deviation. This indicates that dye in significant amounts does not leak out of the pulmonary circulation during its initial circulation despite the high pulmonary capillary pressures bordering on pulmonary edema levels in many of these cases. If dye had left the circulation, outputs calculated by the dye method would have been erroneously higher than those calculated with tagged red cells, which presumably do remain within the vessels.

Dow (18) quotes Ring to the effect that dye outputs exceed tagged cell outputs when the two

indicators are simultaneously injected initially, and that this discrepancy disappears on the subsequent injections in the same dog. The implication that Evans Blue dye is taken out of the pulmonary circulation unless the system is primed or saturated by previous injections was not born out by the results in the present study.

Pulmonary blood volume

It has been proposed by Braunwald, Cournand, and Fishman (19), on the basis of model experiments, that the high flow to volume ratio of the pulmonary bed precludes measurement of the pulmonary blood volume by the Bradley technique. The Bradley formulation is an arithmetic relationship from which, assigning the approximate flow to volume ratio which exists in the pulmonary bed in man, one can readily predict that the mean arteriovenous difference of indicator concentration will be close to 15 per cent of the final equilibration concentration. This implies that an analytical error of one per cent may result in as much as a 6 to 7 per cent error in the final volume. In their model experiments, Braunwald, Cournand, and Fishman (19) used a direct writing densitometer for recording dye concentration. It would seem logical to ascribe an analytical error with this methodology of roughly 5 per cent. This in turn could result in considerably more error in the final pulmonary volume determined by the Bradley formula. The method might well prove valueless under this experimental situation. However, it is to be emphasized that in the experiments reported in this communication, dye analyses for the Bradley volumes were carried out in macrocuvettes on a Beckman DU Spectrophotometer using undiluted plasma and with duplicate checks to well within one per cent. At the same time, the red cell radioactivity was measured sufficiently long to insure less than a one per cent standard error. Under these analytical conditions, it was our feeling that one might well be able to measure the pulmonary blood volume in man with reasonable accuracy despite the relatively high flow to volume ratio.

This hope appears to have been justified by the results shown in Figure 2, which illustrates the relationship between pulmonary blood volumes obtained by the traditional Stewart-Hamilton technique and volumes obtained simultaneously by the Bradley method. A significant correlation between these volumes, $R = +.82$ ($p < .01$), was obtained. The indicator dilution values were generally higher, but this was expected since they included the additional volume of the right ventricle and a portion, if not all, of the right atrium. The overall agreement is satisfactory and would seem to substantiate the fact that the arteriovenous equilibration method is a suitable one for the measurement of the pulmonary blood volume in man. In those situations where cardiac output is markedly depressed, valvular insufficiency is present, or pulmonary blood volume markedly increased, the exponential downstroke of the indicator dilution curve may be difficult to separate from the effects of indicator recirculation. Under these circumstances, extrapolation of the curve may produce considerable error in the determination of pulmonary volume by the indicator dilution method. The Bradley method is at its height of accuracy under these same conditions of low flow or increased volume. Therefore, in these circumstances the Bradley method may actually become the preferable one. It might be noted that the validity of the indicator dilution curve for determination of volume can be established by comparing cardiac output calculated from the same curve

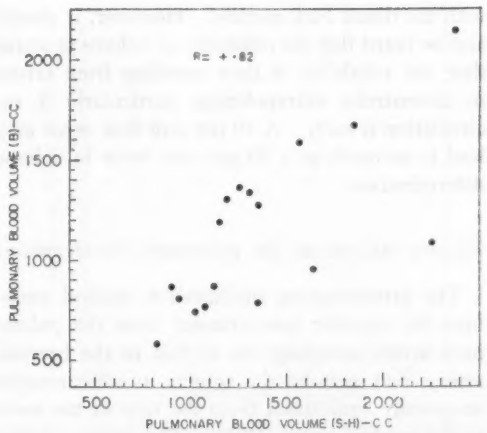

FIG. 2. THE PULMONARY BLOOD VOLUME IN MITRAL STENOSIS

"Pulmonary" blood volumes determined by the Stewart-Hamilton indicator dilution method are compared to volumes simultaneously obtained by the Bradley arteriovenous equilibration method.

with the direct Fick method. However, it should also be noted that the reliability of volume is worse than the reliability of flow resulting from errors in downstroke extrapolation, particularly if recirculation is early. A 10 per cent flow error may lead to as much as a 30 per cent error in volume determination.

Factors influencing the pulmonary blood volume

The arteriovenous equilibration method measures the vascular compartment from the pulmonary artery sampling site to that in the brachial artery, and includes the volume in other arteries temporally equidistant from the base of the aorta to the brachial artery needle. The volume yielded as the product of the mean transit time and cardiac output in these experiments represents the volume of a portion of the right atrium, where injection was made, and the right ventricle, as well as the rest of the volume measured by the arteriovenous equilibration method. Therefore, the difference in mean values obtained by the two methods may in part be explained. Right atrial injection was made necessary by the requirement that blood sampled from the pulmonary artery for the Bradley method be thoroughly mixed with indicator, and thus truly representative of the indicator concentration entering the pulmonary bed. The actual volumes obtained by both the Bradley and the Stewart-Hamilton methods in these patients with

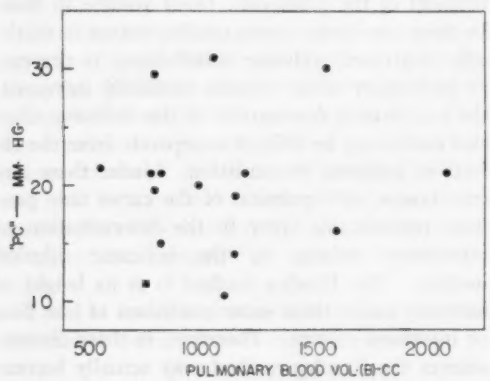

FIG. 3. THE EFFECT OF "PC" PRESSURE ON PULMONARY BLOOD VOLUME

No correlation is seen between "pulmonary capillary" pressure and the "pulmonary" blood volume.

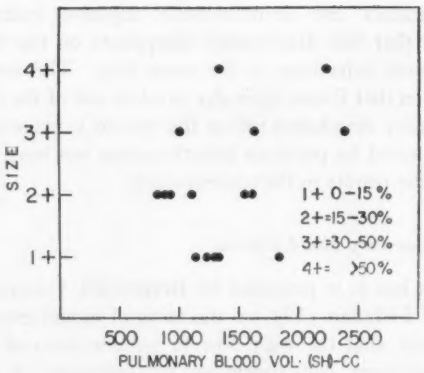

FIG. 4. THE EFFECT OF CARDIOMEGALY ON PULMONARY BLOOD VOLUME IN MITRAL STENOSIS

The absence of correlation between pulmonary blood volume and heart size judged roentgenologically is demonstrated.

mitral stenosis varied over a wide range of values (Table I) with a mean still within the upper range generally seen in normals (6). Since the wide range of values incorporated one or two which were low, many normals, and several which were increased, the hemodynamic data were analyzed to see what factors, in addition to mitral stenosis, might contribute to the volumes in any individual case. Specifically, an attempt was made to correlate the volumes obtained by the two methods with other variables measured by cardiac catheterization.

Figure 3 demonstrates the poor relationship between "pulmonary capillary" pressure and pulmonary blood volume. This confirms the findings of Doyle, Wilson, Lépine, and Warren (7) and of Johnson (20), but is in contrast to the findings of Wade, Werkö, Eliasch, Gidlund, and Lagerlöf (5) who were able to demonstrate such a relationship. On the surface, it would appear difficult to explain the absence of correlation between these two variables, since presumably the increased "pulmonary capillary" pressure in mitral stenosis must be dependent upon either an increase in volume of blood in this bed or an increase in pulmonary venous and left atrial tone. However, when one takes into consideration that the methodology permits detection of volume changes only if greater than 100 cc. or so (7), it becomes apparent that significant changes in volume which could cause increases in pressure might remain undetected.

There was perhaps some tendency for patients with the smallest valve areas calculated by the Gorlin formula (21) to have the larger volumes, but there was no significant correlation, as examination of Tables I and II reveals.

The Stewart-Hamilton method as performed in these experiments included the volume of all the heart chambers, and the Bradley method included the volume of the left heart. It was therefore considered that cardiomegaly in certain individual cases might be predominantly responsible for the volumes which were obtained. Figure 4, however, demonstrates that there was no correlation whatsoever between heart size as judged roentgenologically and the pulmonary blood volumes calculated by the Bradley method. A similar absence of correlation was seen with the Stewart-Hamilton volumes.

Figure 5 illustrates the relationship between the pulmonary blood volume and pulmonary vascular resistance. Despite the three values in the range between 450 and 600 dscm^{-5}, which obviously grossly deviate, the data were found to fit quite well an exponential curve of the general form $\log y = -.3 \log x + 3.75$, where y = pulmonary blood volume and x = pulmonary vascular resistance. The correlation coefficient, derived by the computation of least squares, between

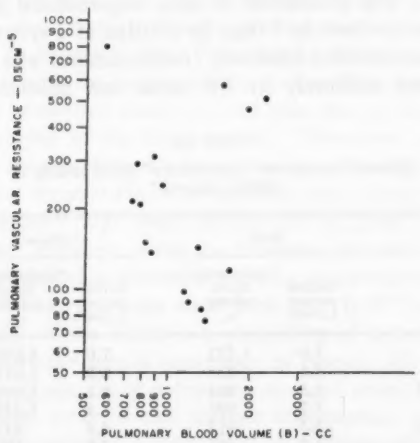

FIG. 5. THE EFFECT OF PULMONARY VASCULAR RESISTANCE ON PULMONARY BLOOD VOLUME

The relationship between pulmonary resistance and "pulmonary" blood volume fits quite well an exponential curve when plotted logarithmically.

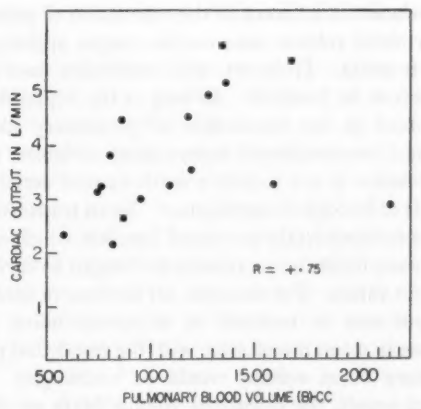

FIG. 6. THE EFFECT OF CARDIAC OUTPUT ON PULMONARY BLOOD VOLUME

A significant correlation is seen between the level of cardiac output expressed as the average of the Evans Blue dye and chromium^{51} values and the "pulmonary" blood volume.

the theoretical variables and the actual measured values was 0.9982. This relationship is quite similar to that noted previously between cardiac output and pulmonary resistance in patients with mitral stenosis (22), as well as in other situations where pulmonary resistance is raised (23, 24). Consequently, it immediately suggested to us that the pulmonary blood volume would be correlated with the level of cardiac output in these patients.

Indeed, a correlation was demonstrated in this study between cardiac output and pulmonary blood volume (Figure 6). The probability of the correlation coefficient of + .75 occurring by chance is less than one in a thousand. A similar correlation was seen between stroke output and pulmonary blood volume. Thus, when cardiac output tended to be maintained at normal levels despite the mitral lesion, pulmonary blood volumes in these cases were in the higher range of normal or increased, whereas in those cases where cardiac output was reduced the pulmonary blood volume tended to be low normal or occasionally reduced. This correlation is in contrast to the findings of Ball, Kopelman and Witham (3) but is in close agreement with similar observations made in a large number of normal subjects by Johnson (20), and also in the group studied by Doyle, Wilson, Lépine, and Warren (7).

Doyle and his associates concluded that such a

correlation is inherent in the calculation of pulmonary blood volume since cardiac output appears in the formula. However, this conclusion does not appear to be justified. As long as the expressions involved in the calculation of pulmonary blood volume are completely independent variables, this correlation is not merely a mathematical certainty but is of biological significance. Mean transit time is an independently measured function which may increase, decrease, or remain unchanged as cardiac output varies. For example, an increase in cardiac output may be matched by a corresponding decrease in mean transit time with the result that pulmonary blood volume would be unchanged. In other words, the calculated volume bears no obligate relationship to any variable used in its determination either with the Stewart-Hamilton or the Bradley formula, and the demonstration of a correlation between pulmonary blood volume and cardiac output would, therefore, appear to have a fundamental significance. Johnson (20) interprets these findings in his normals to indicate that pulmonary blood volume tends to act as a reservoir or blood depot supplying the left ventricle with an increased volume when the needs of the periphery require an increase in cardiac output. We cannot be sure that such a teleological explanation can be accepted, although it is plausible that the pressure volume curve of the capillary-venous bed of the lungs and left heart is such as to require considerable increase in volume in order to cause the small rise in filling pressure of the left ventricle which presumably occurs when the stroke volume of the heart increases. In any case, it would appear clear that the important variable, on which pulmonary blood volume depends, is the cardiac output. Whether pulmonary blood volume will be increased or not in a given patient with mitral stenosis appears predominantly dependent on whether cardiac output is maintained or whether it has been reduced by the processes of mitral valve obstruction and increased pulmonary vascular resistance.

The implication of this relationship between pulmonary blood flow and volume is that the volumes in mitral stenosis, though generally within the normal range, are elevated in respect to the low outputs. To better validate this premise, it would be necessary to know:

1) The pulmonary blood volume in "normals" through the entire range of cardiac outputs, particularly low outputs.

2) How this volume varies with changes in output.

3) What would be the volumes in patients with mitral stenosis if their outputs were higher (normal range).

We have not made a systematic study of this problem as yet, but three separate studies that have been carried out provide some evidence suggesting that the pulmonary blood volume in mitral stenosis is indeed elevated when considered in relation to the cardiac output. This evidence is as follows:

1) Rest and exercise studies of the cardiac output and pulmonary blood volume were determined by the Stewart-Hamilton method and a plasma indicator injected into the pulmonary artery in 7 additional cases of mitral stenosis. The results (Table III) indicate that the volume increased only in those cases (4 patients) in which there was a significant increase in cardiac output. In the 3 patients in whom the output failed to increase appreciably, the volume remained unchanged. The "pulmonary capillary" pressure rose significantly during exercise in all patients regardless of whether or not there was a volume change.

2) The production of acute experimental pulmonary edema in 7 dogs by infusion of a systemic vasoconstrictor substance (methoxamine) was attended uniformly by left atrial and pulmonary

TABLE III

Effect of exercise on "pulmonary" blood volume in mitral stenosis *

	Rest		Exercise	
Pt.	Cardiac output *L./min.*	"Pulmonary" blood volume *cc.*	Cardiac output *L./min.*	"Pulmonary" blood volume *cc.*
Alt.	5.9	1,222	7.0	1,632
Kea.	5.1	733	6.0	1,012
St. P.	4.3	884	8.1	1,064
Bar.	5.7	998	7.4	1,319
O'Co.	5.3	912	5.8	845
Les.	4.2	561	3.8	602
Koz.	3.7	1,387	4.1	1,451

* Cardiac output and "pulmonary" blood volume were studied in seven additional patients with mitral stenosis before and during exercise using Evans Blue dye injected into the pulmonary artery.

venous and capillary hypertension and a markedly reduced cardiac output (25). The alteration of pulmonary blood volume, however, was not uniform, showing no change from control levels in 3, a fall in 2, and an increase in 2. Thus, pulmonary vascular hypertension was not accompanied by a predictable change in volume, a finding qualitatively similar to that of the present study. Similarly, the volumes appeared out of proportion to the very low level of cardiac outputs which averaged but 28 per cent of initial control values.

3) Acute occlusion of one main branch of the pulmonary artery in dogs (26) has no significant effect on the cardiac output, and causes, at most, only slight increases in pulmonary artery pressures; thus the flow through the unoccluded lung essentially doubles. Since the calculated pulmonary blood volume remains constant, it is evident that the vascular volume of the unoccluded lung nearly doubles; therefore, the flow to volume ratio in that lung remains constant. These observations provide evidence for the existence of a relationship between flow and volume. Additional studies of this relationship in animals and humans are clearly indicated and are being considered.

Pulmonary hematocrit

It was observed in all patients that the mean transit time of red cells through the lung is faster than that of plasma. Since the total quantity per unit time of red cells and plasma going into the lung in the steady state is essentially the same as that leaving the lung, it follows that overall pulmonary hematocrit must be lower than that of blood circulating in the large vessels. Therefore, one may say unequivocally that pulmonary hematocrits by the Stewart-Hamilton method were distinctly lower than large vessel hematocrits in these patients. However, when the calculated hematocrits are subjected to statistical analysis, the mean ratio of pulmonary to large vessel hematocrit is .97 (s.d. = .02), which indicates a probability of between 5 and 10 per cent of occurrence by chance. However, calculation of pulmonary to large vessel hematocrit ratio in this manner incorporates any errors in measuring cardiac output and also reflects any errors present in reading the hematocrit of large vessel blood or in correcting it for trapped plasma. These errors are not introduced in the calculation of mean transit times, which become a more accurate reflection of the existing pulmonary hematocrit.

The pulmonary to large vessel hematocrit ratio as computed by the Bradley method had a mean of .91 which was statistically significant ($p < .01$). It is somewhat difficult to explain the significantly lower pulmonary hematocrit by the Bradley method as compared to the Stewart-Hamilton method. It is true that an additional volume of large vessel blood contained in the right ventricle and right auricle which seems to be included in the volume yielded by the indicator dilution method, does not appear in the determination of the pulmonary hematocrit by the arteriovenous equilibration method. However, this in itself seems incapable of altering the mean to this extent, and other limitations and assumptions in both methods, which have been detailed elsewhere (8, 9, 13), may also play a role. In any case, the ability to demonstrate by both these methods that pulmonary hematocrit was less than large vessel hematocrit is of considerable significance, for both methods measure a huge amount of large vessel blood as contrasted to the actual amount of blood in small vessels. Estimates of pulmonary capillary volume (27) indicate that the pulmonary capillary bed probably makes up less than 10 per cent of the total volume measured by these techniques. Therefore, a demonstration of lowered overall hematocrit implies that the hematocrit of the pulmonary capillary bed itself must be very low, undoubtedly something less than 75 per cent of large vessel blood.

These findings contrast with those reported by Lilienfield and his colleagues (28) for normal man, where a significant lowering of pulmonary hematocrit by the Stewart-Hamilton method could not be demonstrated. Our observations are in accord, however, with our findings in normal dogs (29), as well as those of Dow, Hahn, and Hamilton (30) and those of Lawson, Cantrell, Shaw, Blackburn, and Adams (31). It would appear from these results that the pulmonary capillary bed does contribute to the overall lowering of total body hematocrit in comparison to large vessel hematocrit in patients with mitral stenosis. In this series, the ratio of total body to large vessel hematocrit had a mean of .92 with a standard deviation of .04, which is in good agreement with the ratio found by others (32) in man over a wide range of large vessel hematocrits.

SUMMARY

1. The Stewart-Hamilton and Bradley methods for "pulmonary" blood volume determination were simultaneously studied 19 times during right heart catheterization in 16 patients with mitral stenosis utilizing chromium^{51} tagged red cells and Evans Blue dye.

2. No correlation was demonstrated between "pulmonary" blood volume and mitral valve area, heart size, or "pulmonary capillary" pressure. A straight-line relationship when plotted logarithmically between "pulmonary" blood volume and pulmonary vascular resistance was demonstrated, which suggested that another variable related to resistance affected the level of pulmonary blood volume. When the relationship between cardiac output and "pulmonary" blood volume was examined a significant linear correlation was found, suggesting that pulmonary blood flow would appear to be the predominant variable governing "pulmonary" blood volume. Evidence is presented to suggest that "pulmonary" blood volume is elevated out of proportion to the existing blood flow in mitral stenosis, and that it is the presence of a reduced cardiac output in many patients with mitral stenosis which results in a mean "pulmonary" blood volume within the normal range.

3. No evidence of a systematic error in cardiac output was discerned between the two indicator dilution methods themselves or with the direct Fick method. This suggests that Evans Blue dye in significant amounts does not leak out of the pulmonary bed during its initial circulation despite high "pulmonary capillary" pressures.

4. A good correlation was seen between individual volumes calculated by the Bradley arteriovenous equilibration method and those calculated by the traditional Stewart-Hamilton method. Results indicate that with careful analytic technique the Bradley method can be satisfactorily applied to measurement of "pulmonary" blood volume.

5. Average mean transit time was shorter for tagged cells than for dye in all patients. As a result "pulmonary" hematocrit was lower than large vessel hematocrit. Since the methods used measure blood volumes predominantly situated in large vessels rather than in capillaries, the demonstration of a lower "pulmonary" hematocrit indicates that the hematocrit of the pulmonary capillary bed itself must be considerably less.

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THE PHOSPHATE PARTITION OF THE ERYTHROCYTES OF NORMAL NEWBORN INFANTS AND OF INFANTS WITH HEMOLYTIC DISEASE

By TIBOR J. GREENWALT and VALBORG ELIZABETH AYERS

(From the Milwaukee Blood Center, Milwaukee, Wisconsin)

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The mammalian erythrocyte depends on a complex glycolytic mechanism for most of its energy. It is believed that this mechanism controls the transfer of cations across the cell membrane, and serves to maintain the biconcave shape of the red blood cell. Adenosine triphosphate and 2,3-diphosphoglycerate participate in the energy exchanges involved in these processes (1–9).

Alterations in the relationship of the phosphate fractions develop in erythrocytes during storage (10–16). Similar changes do not occur with the senescence of erythrocytes *in vivo* (12). The deviations from the normal pattern may be reversed following the transfusion of stored blood (14), and by the addition of some of the purine nucleosides *in vitro* (17–24). Abnormalities of the metabolism of the erythrocytes in hereditary spherocytosis, auto-immune hemolytic disease with spherocytosis, and in hereditary non-spherocytic hemolytic disease have been demonstrated (25–28). The literature contains only fragmentary data on partitioning of the acid-soluble phosphorus compounds of fetal erythrocytes (2, 29–32). Elevated values have been reported for the inorganic and ester phosphates in the red cells of normal infants (31, 32).

The purpose of this study was to find out if the action of specific antibodies on fetal erythrocytes produces any alteration in the phosphate partitioning. The intra-erythrocytic phosphate pattern of normal fetal red cells was studied and compared with that of the red cells of adults.

MATERIAL AND METHODS

All the infants studied had been delivered normally but were otherwise unselected. There were ten female and six male babies in the normal group and nine females and one male with erythroblastosis fetalis. The average weight of the normal infants was 7.6 pounds (6.1 to 9.0), and the weight of the infants with hemolytic disease averaged 6.8 pounds (5.1 to 8.4). The blood group of one normal infant was A and that of the mother B. The blood group of two infants with hemolytic disease was AB and their mothers were A_2 and B. The other infants were all products of homospecific pregnancies. The medication and anesthesia administered for delivery were similar in the two groups.

The cord blood [1] of the normal babies was drawn aseptically from the umbilical vein at the time of delivery. The blood of the infants with hemolytic disease was obtained at the time of the first replacement transfusion within the first eight hours of life. All of the blood specimens were collected into acid-citrate-dextrose solution (ACD) formula A[2] in the proportion of 100 ml. of blood to 15 ml. of preservative. The final concentration of glucose added was approximately 320 mg. per cent in whole blood. The specimens reached the laboratory within 30 minutes. Aliquots were distributed into sterile vacuum tubes. The chemical analysis of the fresh specimens was started immediately. The remaining aliquots were stored at 4° to 6° C. Mixing of the specimens during storage was avoided in order to duplicate the usual conditions under which "bank blood" is kept. Each specimen was cultured at the time of use, and the data were discarded if contamination was present.

For studying the effect of antibodies added *in vitro* the plasma was removed from a portion of the blood specimen and replaced with sterile anti-D serum [3] and ACD solution. This mixture and the control specimen were incubated at 37 ± 1° C in a water bath, and aliquots of each were distributed into sterile tubes for storage at 4° to 6° C.

Adenosine, 100 μM per ml. of 0.85 per cent sodium chloride, was added to a final concentration of 25 μM per ml. of packed red cells. An identical volume of 0.85 per cent sodium chloride was added to the control specimens. All the solutions were sterile.

A direct Coombs test was performed on each specimen containing antibodies.

Hematocrit determinations were done in duplicate using a high-speed microhematocrit centrifuge.[4]

For phosphate partition the red cells were washed three times with cold saline. The buffy coat was removed after each washing. The inorganic, total, 7-minute hydrolyzable, and 100-minute hydrolyzable phosphate

[1] Cord blood and fetal blood are used interchangeably.

[2] Trisodium citrate 2.20 per cent
Citric acid 0.80 per cent
Dextrose 2.45 per cent

[3] Native serum containing no diluent or preservative.

[4] International Co.

fractions were determined on cold, five per cent trichloracetic acid extracts of the washed, packed red cells by the method of Lowry and Lopez (33). The hydrolyses were conducted for 7 and 100 minutes in 1 N H_2SO_4 at 100° C. The total phosphate was determined after wet ashing the filtrate (34).

The phosphate fractions determined in this manner (12, 15, 35–38) are believed to represent: 1) inorganic phosphate; 2) labile phosphates released by 7-minute hydrolysis mainly from adenosine triphosphate (easily hydrolyzable); 3) phosphates split from adenosine monophosphate and hexose phosphates after 100-minute hydrolysis (difficultly hydrolyzable); 4) total phosphate; 5) largely the phosphates of 2,3-diphosphoglycerate which are estimated by subtracting the first three fractions from the total phosphate (non-hydrolyzable).

Duplicate determinations were performed throughout the study. Analysis of these data reveals:

Fraction*	Number of duplicate pairs	Standard deviation *mg. % P*	Percentage of values falling within	
			1 S.D.	3 S.D.
P_i	96	±0.2506	90.6	98.9
EH	94	±0.3965	86.1	100.0
DH	93	±0.5156	89.2	98.9
Total	94	±1.2153	52.1	93.6

* See legend, Table I.

P values were obtained from t calculated by the formula given by Snedecor (39) for groups of unequal sizes:

$$t = \bar{x}\sqrt{\frac{n_1 n_2(n_1 + n_2 - 2)}{(n_1 + n_2)Sx^2}}$$

where

$\bar{x}$ = the difference between the group means.

Sx^2 = the pooled sum of the squares of the differences from the two group means.

RESULTS

Comparison of the erythrocytes of normal infants and adults

Trend during storage. The data presented in Table I indicate a greater increase in the inorganic phosphorus of the erythrocytes of normal cord blood than in the red cells of adults after storage for three weeks at 4° C. Simultaneously, the easily hydrolyzable and non-hydrolyzable phosphorus fractions of the fetal cells decreased significantly less, and the difficultly hydrolyzable phosphorus decreased appreciably more than in the erythrocytes of adults.

Average values. Comparison of the average values presented in Table I reveals that the inorganic phosphorus fraction (124 μM) of fresh erythrocytes from cord blood is significantly higher than the inorganic phosphorus level (65 μM) of the fresh cells of adults ($p < 0.001$), and the easily hydrolyzable phosphorus fraction in the erythrocytes of cord blood is significantly lower ($p = 0.02$). After storage the relationship of the inorganic phosphorus (934 μM) of the fetal cor-

TABLE I

Comparison of the changes during storage of the phosphate partition of erythrocytes from normal cord blood and from normal adults

Blood sample	Number of individuals	Days stored at 4° C	μM P/100 ml. cells				
			P_i*	EH	DH	Non-H	Total
Normal cord	14	0	124†	277	142	837	1,380
			(73–161)	*(189–431)*	*(31–266)*	*(376–1,424)*	*(900–2,018)*
	10	21	934	225	41	350	1,550
			(734–1,372)	*(185–288)*	*(0–138)*	*(88–588)*	*(1,066–1,861)*
Difference			+810	−52	−101	−487	+170
Normal adult	5	0	65	359	113	1,167	1,703
			(41–82)	*(278–432)*	*(98–137)*	*(950–1,239)*	*(1,448–1,840)*
	7	21	692	218	82	437	1,430
			(632–767)	*(198–264)*	*(28–156)*	*(377–547)*	*(1,324–1,500)*
Difference			+627	−141	−31	−730	−273
p values of differences			= 0.025	< 0.001	= 0.01	= 0.005	> 0.90

* P_i, EH, DH, and Non-H = inorganic, easily hydrolyzable, difficultly hydrolyzable, and non-hydrolyzable phosphate, respectively.

† Numbers not in parentheses represent means.
Numbers in parentheses represent ranges.

TABLE II

Statistical analysis of the differences in the changes in the intra-erythrocytic phosphate fractions which developed during storage of normal cord blood and the blood of infants with anti-D hemolytic disease *

Days stored at 4° C	Pi†	EH	DH	Non-H	Total
3	p‡ = 0.10	$p = 0.70$	$p > 0.90$	$p > 0.10$	$p > 0.30$
7	$p > 0.05$	$p < 0.05$	$p = 0.025$	$p > 0.50$	$p = 0.50$
14§	$p < 0.005$	$p < 0.005$	$p < 0.01$	$p = 0.60$	$p > 0.80$
21	$p = 0.05$	$p < 0.001$	$p > 0.10$	$p > 0.30$	$p = 0.40$

* Increase in P_i and decrease in EH and DH during storage was greater in the erythrocytes of infants with hemolytic disease. Variations in Non-H and Total phosphorus were not significant.
† See legend, Table I.
‡ p = probability value obtained from t. For calculation see methods section.
§ More completely presented in Table III.

puscles to that in the cells of adults (692 μM) remains similar ($p = 0.005$). Analysis of the remainder of the data reveals no significant differences.

Comparison of the intra-erythrocytic phosphate partitioning in normal newborn infants and infants with anti-D hemolytic disease

Trend during storage. The phosphate partitioning in the erythrocytes from aliquots of normal cord blood and of specimens obtained from infants with anti-D hemolytic disease was studied after storage at 4° C for 3, 7, 14 and 21 days. The trend of the changes in the phosphate fractions was observed to be similar after varying periods of storage. The inorganic phosphorus in the erythrocytes from infants with hemolytic disease rose significantly more rapidly than in the erythrocytes from normal cord blood. The easily hydrolyzable and difficultly hydrolyzable phosphate fractions of the cells of infants with hemolytic disease decreased more rapidly than in red cells from normal cord blood. The data of all these observations are not presented. The significance of the progressive differences in the phosphate partitioning which developed during storage of the red cells of the two groups of infants is presented in terms of probability values (p) in Table II. Representative data obtained after 14 days of storage at 4° C are given in more detail in Table III.

Average values. The only significant difference in the phosphate partition of the fresh erythrocytes of the two groups of infants is the higher level of

TABLE III

Comparison of the changes during storage of the phosphate partition of the erythrocytes from normal cord blood and infants with anti-D hemolytic disease

Blood sample	Number of infants	Days stored at 4° C	μM P/100 ml. cells				
			Pi*	EH	DH	Non-H	Total
Normal cord	14	0	124 (*73–161*)	277 (*189–431*)	142 (*31–266*)	837 (*376–1,424*)	1,380 (*900–2,018*)
	8	14	764 (*610–1,015*)	192 (*120–258*)	94 (*0–197*)	421 (*48–667*)	1,472 (*1,061–1,701*)
Difference			+640	−85	−48	−416	+92
Anti-D hemolytic disease	9	0	135 (*112–152*)	389 (*269–510*)	182 (*117–377*)	804 (*373–1,252*)	1,510 (*1,121–2,057*)
	7	14	1,039 (*874–1,181*)	156 (*11–284*)	43 (*0–104*)	440 (*176–747*)	1,677 (*1,441–1,939*)
Difference			+904	−233	−139	−364	+167
p values of differences			<0.005	<0.005	<0.01	= 0.60	>0.80

* See legend, Table I.

TABLE IV

Phosphate partition of normal group O, D positive adult and fetal erythrocytes exposed to anti-D serum in vitro *

Sample	Hours at 37° C	Days at 4° C following incubation	Direct Coombs	μM P/100 ml. cells				
				Pi†	EH	DH	Non-H	Total
Adult—O, D positive								
—unincubated control	0	0	—	116	358	72	1,124	1,670
—+ anti-D, unincubated	0	0	3+	104	292	125	1,002	1,523
—incubated control	3.5	0	—	302	312	108	542	1,263
—+ anti-D, incubated	3.5	0	3+	275	243	177	595	1,290
—stored control	0	21	—	632	198	156	514	1,500
—+ anti-D, stored	0	21	2+	634	200	225	565	1,623
Cord blood—O, D positive								
—unincubated control	0	0	—	131	288	141	745	1,305
—incubated control	21	0	—	639	290	0	764	1,682
—+ anti-D, incubated	21	0	4+	655	170	55	575	1,455
—stored control	0	14	—	820	176	197	505	1,701
—incubated, stored control	1	14	—	713	133	191	529	1,568
—+ anti-D, incubated, stored	1	14	4+	675	191	158	690	1,714

* Adult erythrocytes = erythrocytes of adults.
Fetal erythrocytes = erythrocytes from cord blood.
† See legend, Table I.

the easily hydrolyzable phosphorus found in the infants with hemolytic disease ($p < 0.005$). At 14 days the average inorganic phosphorus content of red cells from infants with hemolytic disease is significantly elevated above that in normal cord blood ($p < 0.005$). No significance can be assigned to the average values for the other phosphate fractions (Table III).

TABLE V

Regeneration of the phosphate partition with adenosine in anti-D hemolytic disease and in normal controls

Sample	Age of specimen at time of analysis	μM P/100 ml. cells				
		Pi*	EH	DH	Non-H	Total
Adult—control stored at 4° C for 25 days	25 days	688	131	117	624	1,562
—+ adenosine at 4° C after 21 days	25 days	39	368	226	924	1,558
Cord blood†—O, D negative						
—fresh control	0.5 hrs.	187	331	154	491	1,163
—incubated 23 hours at 37° C	23.5 hrs.	678	269	58	335	1,339
—+ adenosine at 37° C for 23 hours	23.5 hrs.	140	484	338	410	1,371
—stored 14 days at 4° C	14 days	752	320	57	377	1,506
—stored 14 days, incubated 1 hour at 37° C	14 days	644	302	304	152	1,402
—stored 14 days + adenosine at 37° C for 1 hour	14 days	253	441	333	359	1,386
Cord blood—A, D positive, anti-D hemolytic disease						
—control stored at 4° C for 20 days	20 days	1,150	488	19	381	2,037
—+ adenosine at 4° C after 16 days	20 days	613	424	121	330	1,488
Cord blood—O, D positive, anti-D hemolytic disease						
—fresh control	0.5 hrs.	195	1,351	0	23	1,570
—incubated 23 hours at 37° C	23.5 hrs.	1,203	53	67	295	1,619
—+ adenosine at 37° C for 23 hours	23.5 hrs.	638	207	221	669	1,735
—stored 14 days at 4° C	14 days	1,206	159	89	754	2,208
—stored 14 days, incubated 1 hour at 37° C	14 days	749	254	347	0	1,347
—stored 14 days + adenosine at 37° C for 1 hour	14 days	156	558	266	1,254	2,235

* See legend, Table I.
† Maternal anti-D titer 1:32 in albumin. History of three stillbirths.
ABO groups of all infants compatible with mothers'.

TABLE VI

Phosphate partition of the erythrocytes in other forms of iso-immune hemolytic disease of the newborn

Sample	Age of specimen	Direct Coombs	μM P/100 ml. cells				
			P_I*	EH	DH	Non-H	Total
Anti-c hemolytic disease							
Infant 3 hrs. of age	fresh	3+	260	339	121	484	1,204
Blood stored at 4° C	20 days	3+	1,445	195	186	393	2,219
Anti-B hemolytic disease							
Infant 5 hrs. of age	fresh	(+)	149	230	245	435	1,059
Blood stored at 4° C	15 days	weak	999	286	129	422	1,835
Anti-A hemolytic disease							
Infant 18½ hrs. of age	fresh	1+	103	329	151	1,340	1,922
Blood stored at 4° C	14 days	(+)	765	362	168	230	1,525

* See legend, Table I.

The phosphate partition of the red cells of 11 normal infants and four with hemolytic disease was studied after incubation *in vitro* for one to 24 hours. The appearance of the alterations in phosphate partition was accelerated. The trend was toward higher inorganic phosphorus values in the infants with hemolytic disease, but the data are not sufficiently extensive to permit analysis.

Effect of anti-D antibodies added in vitro

Representative data from experiments in which the blood of D positive adults and infants was exposed to the action of incomplete (blocking) anti-D antibodies *in vitro* are shown in Table IV. Control studies were also performed using the cord blood of an Rh negative infant. Under the conditions employed, the action of the antibodies *in vitro* does not appear to have had any significant effect on the phosphate partitioning of the erythrocytes.

Reversal of the biochemical changes by adenosine

Adenosine was effective in reversing the alterations in phosphate partition produced by storage or incubation in the red cells of normal infants and infants with hemolytic disease. Representative data are given in Table V.

Observations in other varieties of iso-immune hemolytic disease

Phosphate partition studies of the red cells of infants with anti-c, anti-A, and anti-B hemolytic disease which were encountered during the period covered by this study are presented in Table VI. The blood specimens from these babies became available because they required treatment with replacement transfusions. The need for therapy became apparent in the infant with anti-A hemolytic disease because of rapidly deepening jaundice during the first day of life. The clinical and laboratory findings in the infant with anti-B hemolytic disease warranted transfusion treatment right after birth. In these two infants approximately 34 per cent and 83 per cent of the red cells appeared to be spherocytes (*i.e.*, cells having increased thickness /diameter ratio) in stained blood smears.

The increase in the easily hydrolyzable phosphorus after storage of the red cells of the infants with anti-A and anti-B hemolytic disease is an unexpected finding for which no suitable explanation can be given at present.

DISCUSSION

Many differences have been recorded between the erythrocytes of cord blood and those of adults. At the time of birth the red corpuscles are macrocytic and fetal hemoglobin predominates. At least a proportion of the fetal red cells at term have decreased osmotic and increased mechanical fragility (40). Sjölin (41) has shown that the erythrocytes from cord blood lose potassium more rapidly than those of adults when stored at 4° C. He believes that the structure of the fetal red cell membrane may be different. Hollingsworth (42) reported that the erythrocytes of cord blood have a shortened post-transfusion survival. The present study demonstrates that the phosphate partition of the red cells from fresh cord blood differs from that of the fresh red cells of adults. After storage

at 4° C the changes in the phosphate partition of the erythrocytes are not the same in cord blood as they are in the blood of adults (Table I).

It is possible that the deviations in the phosphate partition of fresh fetal cells are due to extrinsic factors such as the anoxia and acidosis at birth, and the anesthesia administered during labor. It is unlikely that the elevated leukocyte and reticulocyte counts present in the specimens used in these studies affected the phosphate partitioning in the erythrocytes to a significant degree. It has been demonstrated by others that leukocyte levels do not have any appreciable effect on the phosphate partition of erythrocytes (13). The same investigators reported higher values for the adenosine polyphosphates and 2,3-diphosphoglycerate in fresh reticulocyte-laden blood, but could not find any difference in the progression of the changes in phosphate partitioning during storage (13). The white cells degenerate during the storage of blood collected with ACD preservative, and do not contribute to the glycolytic activity of stored blood (15). The buffy layer of each specimen used in this study was removed when the specimens were washed.

The easily hydrolyzable phosphorus fraction in the fresh erythrocytes of infants with anti-D hemolytic disease was found to be higher than in normal infants (Table III). During storage the changes in phosphate partitioning in the erythrocytes of infants with hemolytic disease were found to differ quantitatively from those in normal infants (Tables II and III). This indicates that the glycolytic metabolism, in which the phosphate compounds play a major role, is probably altered in the erythrocytes which are coated by anti-D antibodies. The effect of adenosine in restoring the phosphate distribution in these cells toward normal parallels observations which have been reported in hereditary spherocytosis (25–27). The evidence that the reaction between the antigens and antibodies in the ABO blood group system is exothermic may have some bearing on the results of the experiments which have been presented (43). The attachment of anti-D antibodies to the receptors on fetal red cells has been assumed to be the most important factor in the production of this form of hemolytic disease. It remains to be shown why the clinical severity of the hemolytic disease is not regularly correlated with the height of the maternal titer or the degree to which the fetal erythrocytes are coated with antibodies (44). Previous attempts to solve this problem have not been fruitful. Morphologic changes in the red cells are not evident. Spherocytosis is rarely present in uncomplicated anti-D hemolytic disease, and the osmotic fragility is only slightly increased, whereas the mechanical fragility is within normal limits (40). Variations of complement level *in vivo* are probably not a determining factor (45). It is suggested that variations in the metabolic mechanisms, which determine the intracellular phosphate relationships, might supply the answer to this vexing problem. The limited number of patients studied precludes any conclusions at this time even though there seems to be a superficial correlation between clinical severity and the degree of upheaval of the intracorpuscular phosphate relationships. It is not unlikely that similar alterations in the intra-erythrocytic phosphate partition occur in other varieties of isoimmune hemolytic disease of the newborn.

SUMMARY

The phosphate partitioning of the erythrocytes of normal cord blood and of infants with hemolytic disease of the newborn has been investigated.

The inorganic phosphate fraction of fresh red cells from normal cord blood was found to be higher and the easily hydrolyzable phosphorus fraction lower than in the cells of adults. Significant differences in the alteration of the phosphate partitioning of these erythrocytes were demonstrated during storage at 4° C.

A higher level of easily hydrolyzable phosphorus was found initially in the erythrocytes of infants with anti-D hemolytic disease than in normal cord blood. During storage the changes in phosphate partitioning in the erythrocytes of the infants with hemolytic disease were found to differ from those in normal infants.

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THE PRESSOR EFFECT OF THE ANTIDIURETIC PRINCIPLE OF THE POSTERIOR PITUITARY IN ORTHOSTATIC HYPOTENSION [1]

By HENRY N. WAGNER, JR. AND EUGENE BRAUNWALD

(From the National Heart Institute, National Institutes of Health, Bethesda, Maryland)

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Although the antidiuretic property of posterior pituitary extracts is well established, investigators have repeatedly failed to demonstrate a pressor effect of these substances in man (1, 2). Consequently, the hemodynamic effects of posterior pituitary hormones have received relatively little attention. However, it is known that posterior pituitary extracts do produce an increase in arterial pressure after injection into anesthetized or spinal animals (3, 4). The purpose of this report is to present observations on a disease state in man in which it was found that posterior pituitary hormones have a marked effect on arterial pressure.

DESCRIPTION OF PATIENTS

The three patients under investigation had postural hypotension, hypohidrosis, and impotence, a clinical syndrome first described by Bradbury and Eggleston (5).

Patient H. C. (NIH No. 01–16–06) was a 55-year-old former maintenance man. Ten years prior to his admission he began to tire easily. Several months after the onset of these symptoms, the patient had a right inguinal herniorraphy under spinal anesthesia. Following operation, he noted the sudden onset of symptoms related to orthostatic hypotension, impotence and cessation of sweating in the lower half of his body. Since then his disease has been progressive both symptomatically and by objective measurements.

Patient B. B. (NIH No. 01–19–93) was a 66-year-old, retired sea captain. Approximately 15 years ago, a gradual diminution of sweating occurred. Ten years prior to admission, he noted the onset of symptoms of orthostatic hypotension during exposure to high environmental temperatures. His symptoms of heat intolerance, weakness and syncope progressed until one year ago he was forced to retire.

Patient W. A. (NIH No. 00–08–52) was a 66-year-old economist. Eighteen years ago the patient had the sudden onset of severe postural hypotension and impotence. He stated that he has sweated less than other persons throughout his life. Subjectively, he believes that his symptoms have progressed. He had, however, been able to maintain an office job throughout his illness.

[1] Presented before the 48th Annual Meeting of the Society for Clinical Investigation, Atlantic City, N. J., April 30, 1956.

Patients H. C. and W. A. were usually normotensive in the recumbent position. Patient B. B. was usually hypotensive when recumbent, with pressures in the range of 80/60 mm. Hg. Arterial pressures, however, varied greatly with activity and bodily position. In none of the patients was there evidence of diabetes mellitus, amyloidosis, general debility, syphilis, Addison's disease or any somatic neurological disorder.

In all three patients graded passive tilting in the head-up position from 5 to 40 degrees resulted in a progressively lowered arterial pressure, with mild or no associated symptoms. In all patients, passive tilting greater than 45 degrees resulted in weakness, drowsiness, blurring of vision, inability to speak and syncope within several minutes. Arterial pressures fell to levels unobtainable by sphygmomanometry.

Following the fall in arterial pressure associated with passive tilting, a characteristic "overshoot" of the pressure to levels higher than the baseline pressure occurred after the patients resumed the recumbent position. Following the Valsalva maneuver, in none of the patients was there the normal "overshoot" of the arterial pressure to levels higher than the baseline (6).

In patient W. A., an increase of pulse rate of 15 to 20 beats per minute occurred on standing, passive tilting, and during the Valsalva maneuver. In patients H. C. and B. B., no increase in pulse rate occurred with the fall in arterial pressure associated with standing, passive tilting or the Valsalva maneuver. However, during hospitalization nine years ago patient H. C., on standing, developed a tachycardia together with a marked fall in arterial pressure.

In all three patients there was supersensitivity to the pressor effect of norepinephrine. A rise in systolic arterial pressure to levels of 180 to 220 mm. Hg occurred with intravenous infusions of less than one microgram per minute of Levophed®. In all patients, atropine in doses up to 4 mgm. subcutaneously failed to alter the pulse rate.

METHODS AND MATERIALS

During acute studies, arterial pressures were recorded through an indwelling brachial or femoral arterial needle by means of a Statham P 23 A pressure transducer and a Sanborn direct writing oscillograph. Cardiac output was measured by direct Fick principle with the technique of right heart catheterization. Blood gases were analyzed by the method of Van Slyke and Neill (7). Expired gases were analyzed with a Beckman Oxygen

Analyzer, Model E-2, and a Cambridge Carbon Dioxide Analyzer. Effective renal plasma flow was measured by means of the renal clearance of para-aminohippurate (8). Glomerular filtration rate was measured by means of the clearance of inulin (9). Urine was collected by means of an indwelling six-hole rubber urethral catheter. At the beginning and end of each collection period, the bladder was emptied with 40 ml. of air unless the urine volume was greater than 10 ml. per minute. During the studies in which antidiuretic hormone was administered, 2½ to 5 per cent mannitol was infused in an effort to maintain urine volume. The duration of the urine collection periods varied from 15 to 60 minutes.

Total peripheral vascular resistance was calculated according to the formula:

$$\text{Peripheral resistance (dynes sec. cm.}^{-5}) = \frac{1332\ (\text{Mean femoral arterial pressure} - \text{Mean right atrial pressure})}{\text{Cardiac output (ml./sec.)}}$$

The following posterior pituitary preparations were used: highly purified vasopressin and oxytocin,[2] whole posterior pituitary powder, Pitressin® and Pitressin® Tannate in Oil. The purified vasopressin had a concentration of 85 pressor units per milliliter and contained no oxytocin by amino acid analysis. The purified oxytocin had a concentration of 400 units per milliliter expressed as oxytocic units. It possessed approximately 1/1,000 of the antidiuretic activity of the purified vasopressin when studied in four normal persons in the hydrated state.

RESULTS

1. *Effect of posterior pituitary hormones*

Normal subjects. Six normal subjects, ages 18 to 21, were given intravenous infusions of 3.4 to 15.7 units of Pitressin® in saline per hour for a two-hour period. During control studies, on another day, an equal volume of saline was infused at the same rate (3 to 4 milliliters per minute). As observed by previous investigators (1, 2), infusion of Pitressin® had no significant effect on the arterial pressures of these subjects. Single intravenous injections of 500 to 1,000 mU of Pitressin® in 8 normal subjects resulted in a transient elevation of arterial pressure averaging 15 mm. Hg systolic and 18 mm. Hg diastolic, generally lasting 3 to 5 minutes.

Patients with orthostatic hypotension. Administration of Pitressin® and highly purified vasopressin to the patients in the recumbent position resulted in an immediate rise in systolic and diastolic arterial pressure in all three patients. The immediate response to a single injection of 100 mU of vasopressin intravenously in patient H. C. is illustrated in Figure 1. The elevation of the arterial pressure was related to the dose injected. Figure 2 shows the effect of graded intravenous infusions of 1 to 20 mU per minute of highly purified vasopressin on the arterial pressure of patient W. A.

Patients H. C. and W. A. had a significant rise of brachial arterial pressure when given one milliunit per minute of vasopressin by continuous infusion, and following a single intravenous injection of 10 milliunits. Patient B. B. had no elevation of arterial pressure with one milliunit per minute for 15 minutes, but a rise of 100 mm. Hg systolic when infused with 10 milliunits per minute for 10 min-

[2] We are indebted to Dr. Vincent du Vigneaud who supplied the highly purified vasopressin and oxytocin.

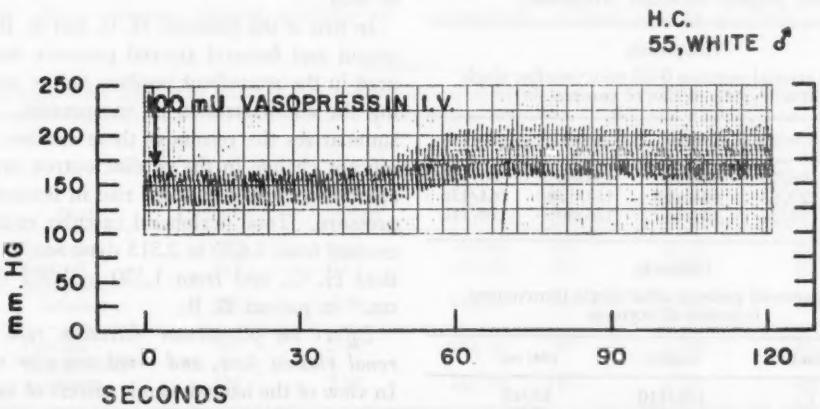

FIG. 1. BRACHIAL ARTERIAL PRESSURE FOLLOWING THE SINGLE INTRAVENOUS INJECTION OF 100 mU VASOPRESSIN IN PATIENT H. C.

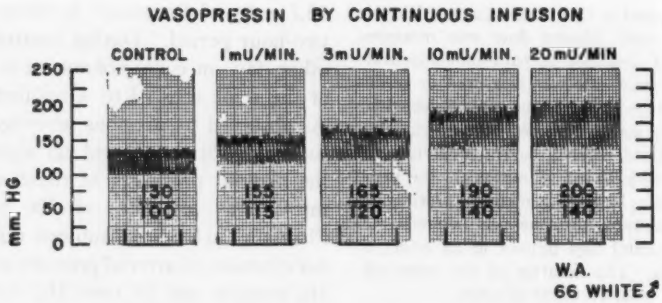

FIG. 2. BRACHIAL ARTERIAL PRESSURE AT 15-MINUTE INTERVALS DURING GRADED INTRAVENOUS INFUSION OF PURIFIED VASOPRESSIN IN PATIENT W. A.

utes. In each patient, arterial pressures in the hypertensive range were observed following the intravenous injection of 1,000 mU of purified vasopressin. The patients had a rise in arterial pressure to sustained hypertensive levels exceeding 170/100 mm. Hg when the following preparations were administered during recumbency: whole posterior pituitary powder by nasal inhalation, Pitressin® by subcutaneous injection, and Pitressin® Tannate in Oil intramuscularly. Table I shows the brachial arterial pressures in each patient following single intravenous injections of vasopressin.

The effects of oxytocin, the second polypeptide of the posterior pituitary, were also studied. In eight normal subjects intravenous infusion of 10 to 20 units of purified oxytocin per hour for two hours had no significant effect on the arterial pressure. Highly purified oxytocin had a marked but transient depressor effect followed by a period of hypertension when administered intravenously to all three patients in the recumbent position. The depressor effect of one unit of oxytocin on one of the patients is illustrated in Figure 3. Table I shows the arterial pressure resulting from a single intravenous injection of one unit of oxytocin in each of the patients.

TABLE I

Effect of single intravenous injections of vasopressin and oxytocin on the brachial arterial pressure of patients with primary autonomic insufficiency

Vasopressin

Average arterial pressure 0–20 minutes after single intravenous injection of vasopressin

Patient	Control	*10 mU*	*100 mU*	*1000 mU*
H. C.	96/70	128/76	158/96	200/130
B. B.	120/98	134/110	152/130	164/136
W. A.	94/58	104/65	126/80	170/110

Oxytocin

Lowest arterial pressure after single intravenous injection of oxytocin

Patient	Control	*1000 mU*
H. C.	138/110	65/45
B. B.	100/76	48/34
W. A.	102/70	50/38

2. Mechanism of the pressor effect of vasopressin

Effect on cardiac output and total systemic vascular resistance. Having demonstrated a pressor effect of vasopressin, an attempt was made to determine if the effect was related to an increase in cardiac output, an increase in peripheral resistance, or both.

In two of the patients, H. C. and B. B., cardiac output and femoral arterial pressure were measured in the recumbent position before and following the administration of vasopressin. Table II summarizes the results of these studies. No significant change in the cardiac output occurred in association with a marked rise in femoral arterial pressure. Total peripheral vascular resistance increased from 1,670 to 2,515 dyne sec. cm.$^{-5}$ in patient H. C., and from 1,350 to 1,975 dynes sec. cm.$^{-5}$ in patient B. B.

Effect on glomerular filtration rate, effective renal plasma flow, and renal vascular resistance. In view of the hemodynamic effects of vasopressin in these patients, an effort was made to determine its possible renal vascular activity. Table III

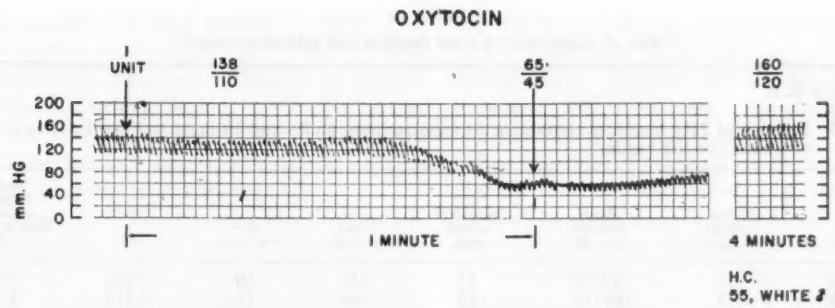

FIG. 3. BRACHIAL ARTERY PRESSURE FOLLOWING THE SINGLE INTRAVENOUS INJECTION OF ONE UNIT OF OXYTOCIN IN PATIENT H. C.

shows the results of a study on patient B. B. It can be seen that there was a fall of the effective renal plasma flow in association with a rise of the systemic arterial pressure evidencing an increase in renal vascular resistance. The glomerular filtration rate was not changed, indicating an increase in filtration fraction. In other studies of patient B. B., and in patients H. C. and W. A., there were similar depressions of the effective renal plasma flow, with a rise in the filtration fraction in association with vasopressin administration. Thus, in each patient renal vascular resistance increased in association with the arterial pressure rise following vasopressin administration.

Effect on pulmonary arterial pressure. In two patients studied, a significant elevation of the pulmonary arterial pressure occurred subsequent to vasopressin administration. The results are shown in Table II.

Effect of ganglionic blockade on response to vasopressin. Four persons without evidence of orthostatic hypotension were given intravenous injections of the ganglionic blocking agent, tetraethylammonium chloride (TEA). In these persons, vasopressin had not previously had a significant pressor effect when injected intravenously. However, when vasopressin was administered subsequent to ganglionic blockade, a pressor response occurred in each subject. This rise in arterial pressure did not occur following control injections of TEA alone. The results of these studies are seen in Table IV.

DISCUSSION

The three patients studied had severe postural hypotension, hypohidrosis and impotence, a clinical syndrome distinct from the postural hypotension associated with general debility, diabetes mellitus, amyloidosis, Addison's disease and various neurological disorders. Involvement of the sympathetic nervous system of these patients is

TABLE II

Effect of vasopressin infusion on cardiac index, systemic and pulmonary arterial pressures and systemic resistance

Pt.	State	O_2 Consumption *ml./min./m²*	Cardiac index *L./min./m²*	Femoral art. press. *mm. Hg* Syst./Diast.	Femoral art. press. *mm. Hg* Mean	Systemic resistance *dynes sec. cm.⁻⁵*	Pulmonary art. press. *mm. Hg* Syst./Diast.	Pulmonary art. press. *mm. Hg* Mean
H. C.	Control 1	121	2.05	96/68	78	1,665	17/10	12
	Control 2	115	2.14	102/70	81	1,670	16/9	12
	Vasopressin* infusion	128	1.95	134/90	110	2,515	22/15	18
B. B.	Control	107	2.85	103/70	84	1,350	21/8	12
	Vasopressin† infusion	124	2.69	145/89	110	1,975	28/15	19

* 1 mU/minute.
† 3 mU/minute.

TABLE III

*Effect of vasopressin on renal function and arterial pressure**

Patient B. B.
Time
9:05 Infusion of 2½% mannitol containing 20 mgm./minute inulin and 10 mgm./minute PAH at a rate of 8.6 ml./minute begun.

Time	Urine volume *ml./min.*	Blood pressure *mm. Hg*	Length of period *min.*	C_{PAH} *ml./min.*	C_{IN} *ml./min.*	F.F.	Renal vascular resistance *dyne sec. cm.*$^{-5}$ $\times 10^{-3}$
10:30	5.4	79/56	15	323	60	0.186	10.3
10:45	7.5	106/74	15	346	75	0.216	13.3
11:00	7.3	106/70	15	323	78	0.242	12.7
11:00	Subcutaneous injection of 5 units of vasopressin						
11:15	4.7	160/96	15	294†	57†		
11:45	2.3	170/100	30	204	68	0.333	31.5
12:05	3.2	164/94	20	222	75	0.338	27.5
12:25	3.9	164/92	20	197	69	0.350	30.7

* C_{IN} = Inulin clearance.
C_{PAH} = Para-aminohippurate clearance.
F.F. = Filtration fraction.

† These data have been discarded. Volume changes occurred that made accurate determinations of inulin and PAH impossible because of delay time and dead space.

indicated by the diminished sweating, postural hypotension and absence of hypertensive "overshoot" following Valsalva maneuver (10). However, all features of the syndrome cannot be explained by a lesion of the sympathetic nervous system only. Abnormalities of the parasympathetic nervous system in all three patients were suggested by: 1) the failure of atropine to alter the pulse rate; 2) the absence of bradycardia during administration of norepinephrine and neostigmine; 3) the lack of effect on the pulse rate and arterial pressure of carotid sinus massage; 4) the inability to have erections; 5) the failure of hypoglycemia to produce an increase in gastric acidity.[3] Thus, this disease, which may be called Primary Autonomic Insufficiency, appears to be diffuse, involving portions of the parasympathetic as well as the sympathetic nervous system. The syndrome in our three patients is most closely simulated in normal man by ganglionic blocking agents.

In these patients with diffuse autonomic nervous system disease, vasopressin in very small doses resulted in a pressor response and significant hemodynamic changes. Since one milliunit per minute of vasopressin approaches the physiologic range of antidiuretic hormone secretion (11), it is possible that endogenous antidiuretic hormone may have a significant hemodynamic effect. Attempts to evaluate the hemodynamic effects of endogenous antidiuretic hormone have thus far

[3] The hypoglycemia study was conducted in only two patients.

TABLE IV

*Effect of vasopressin on arterial pressure during ganglionic blockade**

Subject	Control B.P.	Average B.P. 0.5–20 min. after 300–400 mgm. TEA I.V.	Control B.P.	Average B.P. 5–20 min. after 500 mU Pitressin® I.V.	Control B.P.	Average B.P. 5–20 min. after 500 mU Pitressin® after TEA
Y. S.	120/80	118/79	130/85	125/94	122/80	158/108
G. E.	106/58	101/61	120/64	131/82	110/64	138/102
M. G.	132/80	128/80	132/82	140/87	132/82	156/106
M. U.	180/90	183/95	170/80	170/82	188/92	228/110

* The administration of Pitressin® alone, tetraethylammonium chloride (TEA) alone, and Pitressin® administered 5 minutes after TEA was observed on separate occasions. Arterial pressures were measured by sphygmomanometry at one-minute intervals. Control periods were at least 20 minutes.

been unsuccessful in these patients. Administration of nicotine acid tartrate (12) failed to elicit any antidiuretic response. Changes in plasma osmolality (as in dehydration) resulted in extracellular fluid volume changes which in themselves were associated with pronounced effects on systemic arterial pressure in these patients.

The pressor activity of vasopressin in these patients is possibly related to supersensitivity of the vascular smooth muscle (12, 13) and/or the diminution of normal vasoregulatory reflexes which may counteract the vasoconstricting effect of vasopressin in normal persons. The importance of the latter factor is suggested by the finding that these patients have an exaggerated arterial pressure response to various vasoactive substances, such as norepinephrine and oxytocin. The fact that plasma volume changes, such as those associated with rapid withdrawal of blood and intravenous infusions of albumin and saline, have marked effects on the arterial pressure also supports the concept that vasoregulatory mechanisms are markedly deficient.

A number of workers have shown that the pressor action of epinephrine, norepinephrine, angiotonin and renin is greatly increased after ganglionic transmission is blocked by tetraethylammonium chloride (14, 15, 16). The present observations of the pressor action of vasopressin in the absence of normal function of the autonomic nervous system suggest that autonomic nervous system activity may normally condition the hemodynamic response to the antidiuretic principle of the posterior pituitary as well as to these other substances. The present studies revealed that vasopressin has a pressor effect when given to normal man during chemical ganglionic blockade.

The finding that vasopressin has a marked pressor effect in patients with this type of postural hypotension led to an evaluation of the therapeutic value of vasopressin in these patients. Pitressin® Tannate in Oil has been repeatedly administered to one patient. Following his morning injection he obtained complete relief of his postural symptoms throughout the entire day.

The other two patients have obtained less but definite subjective and objective relief of hypotensive symptoms following intramuscular Pitressin® Tannate in Oil and whole posterior pituitary powder by nasal inhalation. In all patients during vasopressin therapy, a postural fall in arterial pressure was still noted, but, in contrast to the untreated state, the pressure did not fall to levels associated with symptoms. No effect on impotence or anhidrosis was observed.

Two potential complications of vasopressin in therapy must be considered. In patients with coronary artery disease, vasopressin in large doses may result in myocardial ischemia. In our patients no electrocardiographic changes occurred, nor was there any clinical evidence of angina pectoris. Nevertheless, this potential hazard of the use of vasopressin must be emphasized (17). A second possible complication is water intoxication which can be avoided by restricting fluid intake.

SUMMARY

1. Three patients with the syndrome of idiopathic postural hypotension, hypohidrosis and impotence were studied.

2. Although antidiuretic substances from the posterior pituitary had no significant effect on the arterial pressure of normal man, administration of various posterior pituitary hormones to these patients had a pronounced effect on systolic and diastolic arterial pressures.

Hypertension in the recumbent position was produced in all patients by whole posterior pituitary powder administered by nasal insufflation, intra-muscular injection of Pitressin® Tannate in Oil, and subcutaneous and intravenous administration of highly purified vasopressin. Administration of purified oxytocin resulted in marked but transient hypotension.

3. In the two patients studied, the pressor response was produced by an increase in peripheral vascular resistance without significant change in the cardiac output. Vasopressin resulted in a significant increase in pulmonary arterial pressure and in renal vascular resistance.

4. In normal subjects the administration of vasopressin subsequent to ganglionic blockade with tetraethylammonium chloride resulted in a significant elevation of arterial pressure.

5. Vasopressin has been of value in the management of orthostatic hypotension of this type.

ACKNOWLEDGMENT

We are indebted to Dr. Roger K. McDonald for his cooperation in this study.

Addendum

Since preparation of this manuscript, the observations of the marked pressor effect of vasopressin have been confirmed in two additional patients with the syndrome of primary autonomic insufficiency.

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RHEOLOGIC STUDIES ON SYNOVIAL FLUID

By MILTON G. LEVINE and DAVID H. KLING

(From the Boyar-Kling Arthritis Clinic, Los Angeles, Calif.)

(Submitted for publication March 28, 1956; accepted September 5, 1956)

The relative viscosity of normal and pathological synovial fluids fluctuates over a wide range, and has been studied for a considerable period. The older literature is covered by Kling (1), and the more recent by Ropes and Bauer (2) and by Sundblad (3). Early investigators established, by precipitation reactions with acetic acid, that the high viscosity was due to a "mucin," assumed to be a combination of protein and carbohydrate. In 1939, Meyer, Smyth, and Dawson (4) identified the polysaccharide as hyaluronic acid, and found it to be responsible for the high viscosity.

Further studies revealed that solutions of hyaluronic acid in common with other macromolecular substances exhibited non-Newtonian viscosity. Therefore, relative viscosity, heretofore the only viscosity factor determined, was no longer sufficient to characterize the rheological behavior of synovial fluid. Other types were investigated, including intrinsic and anomalous viscosity.

In the following studies we describe a simple capillary viscometer which can be used to measure relative, intrinsic and anomalous viscosity.

Results are presented of viscosity determinations in synovial fluids from normal joints of animals and pathological effusions from various types of human arthritis.

DESCRIPTION OF VISCOMETER

Our instrument (Figure 1) is a modification of the capillary viscometer described by Mann (5) for measuring plasma fluidity. The Mann, like the standard Ostwald viscometer, cannot be used conveniently to measure anomalous viscosity because it is constructed to operate at a fixed stress. By substituting a graduated pipette for the volumetric pipette suggested by Mann we have been able to measure the anomalous viscosity under variable stress.

In most instances we used a one-ml. pipette, graduated in 0.1 ml. The length of the pipette from zero point to tip was about 23 cm. Other calibers and lengths of the pipette may be selected. The capillary consisted of a spinal or hypodermic needle about 10 cm. in length attached to the delivery end of the pipette. The length and gauge of the needle may be varied. A 20 gauge is suitable for most joint fluids; a 22 gauge is preferable for fluids of low viscosity, and an 18 gauge for highly viscid fluids.

The attachment of the needle to the pipette is made water-tight by a rubber sleeve, or by a cement seal, or the delivery end of the pipette may be ground so that it fits snugly into the hilt of the needle.

The viscometer is filled by suction above the zero mark, and the upper opening of the pipette is kept closed with a finger. The pipette is placed in a clamp holder and immersed in a constant temperature bath until the hilt of the hypodermic needle meets the water level. The measurement is started by releasing the finger, and the rate of flow is timed between two selected points on the pipette, either by means of two stop watches or an electronic recorder. Readings are made at close to room temperature. The water level is not appreciably altered by the small amount of synovial fluid which flows into the bath. The metal capillary adjusts almost instantaneously to the temperature of the water bath. Differences between the temperature of the synovial fluid in the pipette and the temperature of the water bath have only a slight effect on the efflux time as shown in the following experiment:

With the water bath temperature kept constant at 21.5° C the temperature of the fluid in the pipette was varied between 17° C and 37° C. At 17° C the average efflux time for 10 determinations was 15.4 seconds; at 27° C the average time was 15.2 seconds; at 37° C the average time was 14.9 seconds. In this severe test of the effect of the temperature of the fluid in the pipette on the efflux time the variations were minor over a temperature range of 20° C. In actual practice the temperature of the fluid and the temperature of the water bath are kept close together, so that the temperature error is negligible. The experiment quoted was representative of three which gave similar results.

If the rate of shear is measured for every 0.1 ml. of fluid volume, from zero to 0.9 ml., the first pressure will be an average of: h_1—the distance from the exit point of the capillary to the zero point on the pipette, and h_2—the distance from the exit point of the capillary to 0.1-ml. mark on the pipette.

With every drop of 0.1 ml., which corresponds to about 2 cm. in length on the pipette, there is a corresponding decrease in the average pressure head. The unit volume may be varied as well as the pressure head. This may be done by changing the distance between h_1 and h_2.

A constant temperature is maintained in the bath with a thermostatically controlled heating unit. It is best not to use a mechanical stirrer, which may disturb the water level and increase turbulence at the end of the capillary.

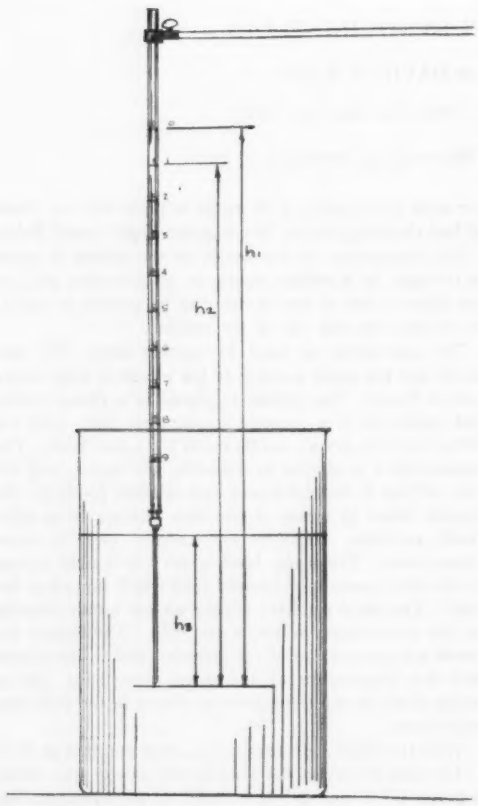

FIG. 1. SIMPLE CAPILLARY VISCOMETER

If the efflux time is determined between the zero point and the 0.1-ml. mark on the graduated pipette, the average pressure head (p) for a fluid of density (d) will be:

$$p = d\left[\frac{(h_1 + h_2)}{2}\right] - h_3.$$

A gallon jar filled with water of the desired temperature will keep a constant temperature for several hours.

Repeated determinations of the efflux time vary only within 0.3 of a second. Proper cleaning is of great importance because dust or other particles may partially obstruct the pipette and needle and change the efflux time. In contrast to most capillary viscometers, cleaning is very easily done by attaching the pipette to a suction pump and rinsing with distilled water and alcohol, and drying with ether. The metal capillary is unbreakable. The kinetic energy correction is small in this viscometer, less than 1 per cent, and has been neglected in the calculations.

In the determination of relative viscosity in any single viscometer, all factors are constant except the efflux time and density. To obtain the relative viscosity it is necessary to compare the flow time of the solution with the flow time of the solvent, which in the case of synovial fluid is water. In our material the density of the synovial fluids varied from 1.012 to 1.025, with an average of 1.019, and could be neglected.

Based on Poiseuille's formula, the relative viscosity of synovial fluids measured by our viscometer is expressed as: $\eta \text{ rel} = \frac{d\,t\,s}{t\,w}$. Where $t\ s$ is the time of efflux of the synovial fluid, $t\ w$ the flow time for water, and d is the density of synovial fluid.

Any of a number of pressure heads may be selected to determine relative viscosity. We used an average hydrostatic pressure of approximately 12.8 cm. of water, which corresponded to the 0.5-ml. and 0.6-ml. gradation on the pipette used in these studies. The efflux time of 0.1-ml. water was 2.8 seconds. The length of the capillary was 9.9 cm., the diameter 0.29 mm. The length of the pipette from tip to zero point was 23.2 cm., the bore was 3 mm., and the temperature of the water bath was 29° C.

MATERIAL AND METHODS

Synovial fluid from normal human joints was not available to us. Therefore, synovial fluid was aspirated from the carpal joints of horses and cattle obtained from the packing house immediately after slaughter. After aspiration, the joints were opened. Fluid was used only from joints with grossly normal articular cartilage and synovial membrane. Roentgenograms showed the articular surfaces to be intact. Microscopic sections of the synovial membrane of these horses and cattle were normal, except that in the former, slight edema and hyperemia were frequently present. Pathological effusions were aspirated from the knee joints of patients with rheumatoid arthritis, osteoarthritis (degenerative) and chronic traumatic synovitis.

Viscosity determinations were done after the fluid was freed from suspended cells and other particles by prolonged centrifugation. If the viscosity tests could not be run immediately, the fluid was kept in the refrigerator at 4° C. No changes of viscosity occurred even after prolonged storage.

The hyaluronic acid concentration was determined by a combination of the turbidimetric methods described by Meyer (6) and by Schmith and Faber (7). Ragan and Meyer (8) found that the turbidimetric method is satisfactory for the determination of hyaluronic acid in synovial fluid. The method is based on the fact that partially depolymerized hyaluronic acid at a pH of about 4.2 produces a colloidal suspension in the presence of serum protein (9). Synovial fluid was treated with a minimal amount of hyaluronidase to assure uniform turbidity. The amount used was determined by titration. Serum reagent was prepared by the method of Schmith and Faber (7), frozen, thawed out before use, and heated at 99° C for ten minutes as recommended by Tolksdorf, McCready, McCullagh, and Schwenk (10).

The steps of the determination were as follows:

In a test tube
Add 0.1 ml. of synovial fluid to 0.8 ml. of 0.85 per cent sodium chloride

TABLE I

Relative viscosity of synovial fluid *

Species	Joint	Diagnosis	No.	Volume, *ml.* Range	Volume, *ml.* Average	Viscosity Range	Viscosity Average
Cattle	Carpal†	Normal	40	2–10	5.	9–10,300	929
Horse	Carpal	Normal	25	2–12	6.	4.5–119	16
Human	Knee	Osteo-arthritis	87	1–120	32.	10–2,830	106
Human	Knee	Rheumatoid arthritis	168	2–200	29.	2–83	18
Human	Knee	Chronic traumatic synovitis	32	4–90	32.	2–68	20

* The relative viscosity of some pathological effusions was determined by Mann and other capillary viscometers which gave values in the range of our instrument.

† This is a composite articulation of three joints in the foreleg: 1) the radio-carpal joint; 2) the intercarpal joint; 3) the carpo-metacarpal joint. These joints are equivalent to the wrist joint of humans, but are usually referred to as knee joints of animals. The fluids were obtained from the two proximal joints.

Add 0.1 ml. of hyaluronidase solution previously titrated
Incubate at 37° C for thirty minutes
Add 1 ml. of buffer solution pH 6 (0.1 M sodium acetate and 0.15 M sodium chloride)
Add 8 ml. of diluted serum reagent made by diluting serum reagent with 0.5 M sodium acetate buffer pH 4.2 dilution of 1:4
Incubate at 30° C for thirty minutes and read the turbidity in a Coleman spectrophotometer at 580 mμ

Serum reagent is prepared as follows:
Horse serum is diluted 1:10 with 0.5 M sodium acetate buffer pH 4.2. The pH is adjusted to 3.1 by the addition of 4 N HCl.
The mixture is frozen. Before use it is thawed out and heated at 99° C for ten minutes and then diluted 1:4 with 0.5 M sodium acetate buffer.

The turbidimetric method gave reproducible curves within 5 per cent. The turbidities disappeared after exposure to an excess of testicular and streptococcal hyaluronidase. The latter attacks only hyaluronic acid; therefore, this is evidence that hyaluronic acid and no other mucopolysaccharide was determined by the turbidimetric method. Furthermore, electrophoretic patterns of normal and pathological synovial fluids have shown boundaries characteristic only for mucopolysaccharides of a mobility in the range of hyaluronic acid (11).

Total and differential cell counts, icterus index, pH, total protein, albumin and globulin, uric acid, bacterial cultures, guinea pig inoculations, serological and other tests were performed on part of the material.

RESULTS

Relative viscosity was determined in 65 normal fluids from animals and 287 pathological effusions (Table I). The highest values, and also the widest range of viscosity, were obtained from the normal fluids of the carpal joints of cattle. Fluid from the carpal joints of horses showed a markedly narrower range and a much lower average relative viscosity. Sundblad (3) found very low relative viscosities from 3 to 29 in 10 fluids from normal astragalotibial joints of horses. He ascribed the low values to dilution with plasma as evidenced by large volumes ranging from 5 to 40 ml. However, in our material the volume in both cattle and horse joints was fairly small and the range was almost identical (2 to 10 and 2 to 12 ml.).

Several factors must be considered to explain the discrepancy between the relative viscosity of the fluids from normal carpal joints of cattle and horses. First, there is an age difference. The age of cattle ranged from 10 months to 3 years, whereas the horses averaged 8 years. Second, edema and hyperemia in some of the synovial membranes of the horses have led to exudation. Even small dilutions of 5 per cent to 10 per cent can cause an enormous fall in relative viscosity, as first pointed out by Schneider (12). This is especially true of normal fluids with highly polymerized hyaluronic acid. Other contributing factors will be taken up later in the discussion.

The relative viscosity of the pathological effusions from osteoarthritis had the widest range and the highest average values. On the other hand, the effusions in rheumatoid arthritis and chronic traumatic synovitis had significantly small ranges and lower average values.

Intrinsic viscosity

Intrinsic viscosity $[\eta]$ is the limiting value of the ratio $\frac{(\eta\ s\ p)}{c}$ at zero concentration. For the

calculation of the intrinsic viscosity, we used the derivation of Huggin's formula originally suggested by Schulz and Blaschke (13) and employed by Sundblad (3) for determining the viscosity of synovial fluid:

$$[\eta] = \frac{\frac{(\eta\, s\, p)}{c}}{1 + k'(\eta\, s\, p)}.$$

The specific viscosity ($\eta\ s\ p$) is equal to the relative viscosity minus one. In this formula k' is approximately constant for members of a homologous series of polymers in a given solvent. It can be found experimentally by first determining intrinsic viscosity by extrapolation. Sundblad (3) reported the average value for k' to be about 0.16 for hyaluronic acid from normal and pathological fluid. Substituting 0.16 for k' and knowing the concentration, the intrinsic viscosity may be determined merely by obtaining the specific viscosity. Sundblad found this to be true for specific viscosities of from 0.3 to 15.0.

Thus it is possible to dilute fluids to an arbitrary specific viscosity below 15, and by determining the concentration of hyaluronic acid at that dilution, the intrinsic viscosity may be derived from Sundblad's formula.

The results of hyaluronic acid determinations and the intrinsic viscosity on 54 animal and 56 pathological effusions are given in Table II. The values for both intrinsic viscosity and hyaluronic acid concentration fluctuate in a considerable range. The highest average intrinsic viscosity (43.8) occurred in synovial fluid from normal carpal joints of cattle. The average values from the same joint of the horses were lower (36.1). For the pathological effusions, the average intrinsic viscosities in osteoarthritis (35.9) and in traumatic synovitis (33.3) were higher than in rheumatoid arthritis (29.1). Since the intrinsic viscosity reflects the mean polymerization, it is observed that the most highly polymerized hyaluronic acid was found in the normal synovial fluid from cattle, and the lowest in effusions from rheumatoid arthritis.

The fluids of the normal carpal joints of the cattle show a high average hyaluronic acid concentration (200 mg. per cent), while in the same joint of the horses the values were lower (136 mg. per cent). Sundblad (3) gives the following values for 11 normal calves: volume one to three ml., hyaluronic acid concentration 193 mg. per cent. This is in the range of our own values of the joints in young cattle. Only in one ox did Sundblad find a low value of 45 mg. per cent. The volume of the fluid here was high, namely, 30 ml.

In osteoarthritis the average concentration of hyaluronic acid (214 mg. per cent) is greater than in rheumatoid arthritis and chronic traumatic synovitis (158 mg. per cent and 148 mg. per cent, respectively). Higher concentrations of hyaluronic acid in osteoarthritis than in rheumatoid arthritis were also found by Fletcher, Jacobs, and Markham (14). Sundblad (3), on the other hand, reported the concentration of hyaluronic acid in osteoarthritis to be similar to that in rheumatoid arthritis.

Anomalous viscosity

Anomalous viscosity of synovial fluid has been investigated with the Couette viscometer by Fessler, Ogston, and Stanier (15), or with the horizontal capillary viscometer with or without manostat controlled pressure by Johnston (16), Scott Blair, Williams, Fletcher, and Markham (17), and Sundblad (3). Ostwald and Auerbach (18) have shown that anomaly can be meas-

TABLE II
Intrinsic viscosity and hyaluronic acid concentration of synovial fluid

Species	Joint	Diagnosis	No.	Intrinsic viscosity	Concentration hyaluronic acid mg. %
Cattle	Carpal	Normal	30	43.8 ± 3.9*	200 ± 57
Horse	Carpal	Normal	24	36.1 ± 7.8	136 ± 77
Human	Knee	Osteoarthritis	16	35.9 ± 8.7	214 ± 91
Human	Knee	Rheumatoid	32	29.1 ± 5.4	158 ± 60
Human	Knee	Chronic traumatic synovitis	8	33.3 ± 8.4	148 ± 86

* Standard deviation.

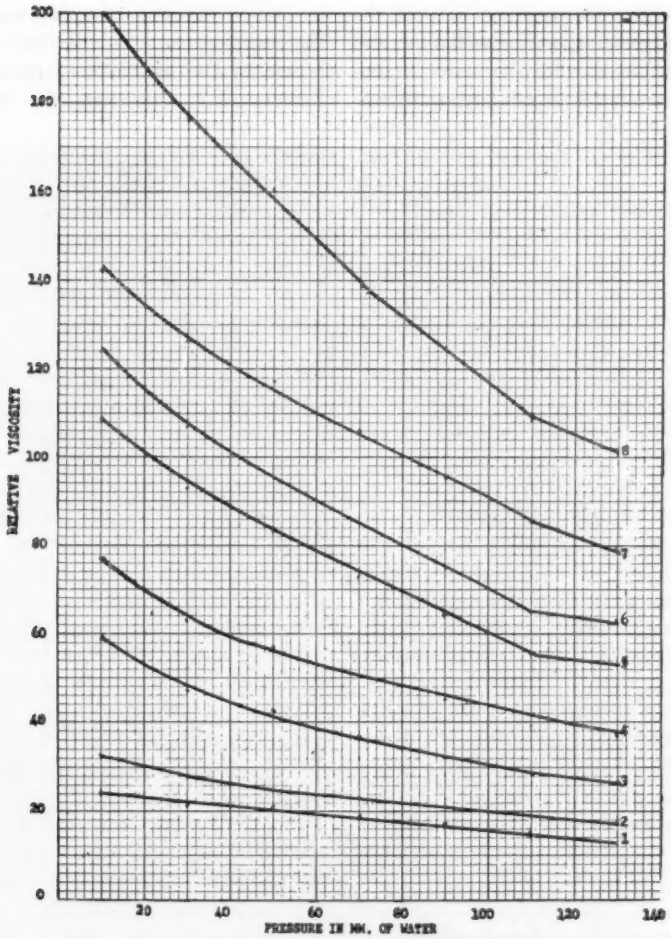

FIG. 2. ANOMALOUS VISCOSITY

The relative viscosity varies with the hydrostatic pressure forcing the liquid through the capillary. The lower three curves are of synovial fluid from patients with rheumatoid arthritis. The upper five curves are of synovial fluid from patients with osteoarthritis. The curves demonstrate the increase in anomalous viscosity with increasing relative viscosity.

ured accurately in a vertical capillary viscometer, using gravity alone or with added pressure to vary the stress. Zamboni (19) has demonstrated that the peak of anomaly for extracts of "mucin" from synovial fluid and umbilical cord occurs at hydrostatic pressures below 10 cm. of water. The dimensions of both the capillary and the pipette employed in our instrument fall within the range generally regarded as satisfactory for viscosity measurements of hyaluronic acid.

In our viscometer, anomaly is measured by varying the hydrostatic pressure. As the height of the column of fluid falls, the hydrostatic pressure decreases and the relative viscosity increases. The gradations on the pipette of our viscometer correspond to 0.1 ml. in volume. For the whole pipette, which holds one ml., there are ten possible points at which the efflux time can be measured. We have read the points corresponding to 0.1 ml., 0.2 ml., *etc.*, down to 0.9 ml. The greatest ac-

curacy lies in the efflux time readings between the points on the pipette corresponding to 0.3 ml. and 0.8 ml. If we plot flow time for this series of consecutive points converted into centimeters of water pressure by measuring the height of the column, the resulting curve is indicative of the rate of increase of the anomalous viscosity (Figure 2). This curve is dependent upon two variables, the

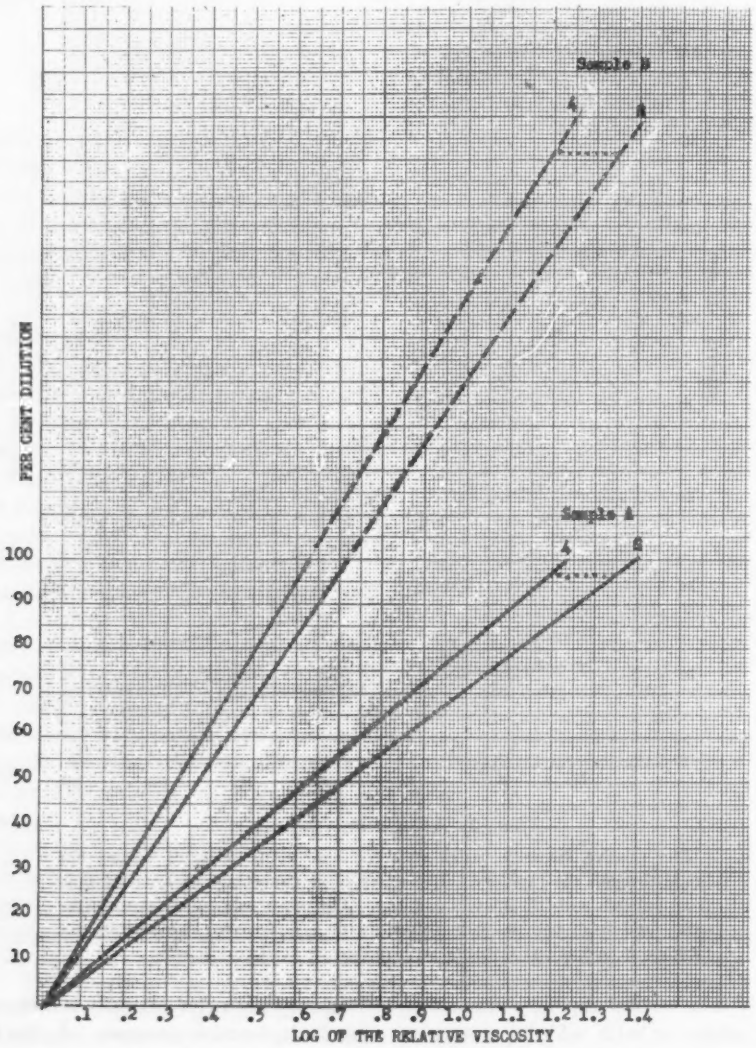

FIG. 3. CURVES FOR DETERMINING THE ANOMALY FACTOR

The reference point is the log 1.2 for the relative viscosity at the 4th point of stress (corresponding to a pressure of 170 mm. of water). The dilution which gives this relative viscosity is then found on the curve for the 8th point of stress (corresponding to a pressure of 85 mm. of water). The difference between the logs of the relative viscosities at the two points of stress is the anomaly factor. In the figure the dotted line indicates this difference. Samples "A" and "B" are identical in polymerization, but "B" contains half the concentration of hyaluronic acid of sample "A." Both samples give the same anomaly factor (0.15).

concentration and polymerization of hyaluronic acid in the synovial fluid. For the anomaly to reflect the degree of polymerization alone, the effect of concentration of hyaluronic acid must be eliminated.

Our method for doing this is based on the fact that the logarithms of the relative viscosities of a series of dilutions of synovial fluid give nearly a straight line when plotted against per cent dilution. If this is done for two different points of stress on our viscometer, two straight lines result which converge near the point of infinite dilution. We have arbitrarily chosen the logarithm of one particular relative viscosity at one point of stress as a reference point. The increase in the viscosity which occurs at a second point of stress for the same dilution is indicative of the increase in the degree of anomaly. To illustrate that this occurs independent of the concentration, we have tested two samples of fluid (Figure 3). One fluid has been diluted to 50 per cent of the concentration of hyaluronic acid of the first. The degree of polymerization is the same. The reference point is the log 1.2 for the relative viscosity at the fourth point of stress which corresponds to an average hydrostatic pressure of approximately 170 mm. of water. In sample "A" this corresponds to a dilution of 97 per cent. For the same dilution at the eighth point of stress with average hydrostatic pressure, corresponding to 85 mm. of water, the logarithm is found to be 1.35. The difference between the logarithms at the eighth and the fourth points of stress is equal to 0.15. This we have labelled the anomaly factor.

In sample "B" which has half the concentration of hyaluronic acid of "A," the reference point must be the same as for sample "A." In order to obtain this value the curve for the fourth point of stress is extrapolated to cross the reference point η rel logarithm 1.2. In like manner, the curve for the eighth point of stress is also extrapolated. At the dilution where the fourth point of stress crosses the line for log 1.2 the corresponding point for the eighth point of stress is log 1.35. The difference, or anomaly factor, again is 0.15. In other words, regardless of the initial concentration, this method for determining anomalous viscosity gives the same anomaly factor, which then may be considered directly proportional to the degree of polymerization.

It must be stressed that we have arbitrarily chosen the reference point as η rel log 1.2. Any other point is just as good, providing it is used consistently. Fluids diluted to a relative viscosity below 20 are preferable for this type of determination because in highly viscid fluids the straight line tends to curve.

The intrinsic viscosity is chiefly an index of mean polymerization of hyaluronic acid. According to Sundblad (3), the degree of anomalous viscosity is more sensitive to the presence of small amounts of high polymer fractions in pathological fluids, whereas in highly degraded fluids the intrinsic viscosity is more reliable.

Table III lists the average anomaly factors for 33 normal fluids from the carpo-metacarpal joints of cattle and horses and 39 effusions from three types of arthritis. The highest values were found in the fluids from the cattle. The fluid from the horses had lower values, although there is an overlap between these two groups.

Of the pathological fluids, osteoarthritis (degenerative) has higher values than rheumatoid arthritis and chronic traumatic synovitis. Here again there is an overlap. The analysis of results indicates higher polymerization of hyaluronic acid in normal synovial fluid from cattle and horses than from pathological human effusions. In osteoarthritis (degenerative) the average degree of polymerization is higher than in rheumatoid arthritis and traumatic synovitis.

DISCUSSION

With the simple capillary viscometer described, we have determined relative, intrinsic and anomalous viscosity.

Relative viscosity, the simplest and most frequently used parameter, is not only important in

TABLE III

Anomalous viscosity of synovial fluid

Species	Joint	Diagnosis	No.	Average anomaly factor
Cattle	Carpal	Normal	20	.281 ± .068*
Horse	Carpal	Normal	13	.227 ± .04
Human	Knee	Osteoarthritis	10	.177 ± .034
Human	Knee	Rheumatoid	20	.122 ± .034
Human	Knee	Chronic traumatic synovitis	9	.137 ± .035

* Standard deviation.

itself, but is also necessary for the determination of intrinsic and anomalous viscosity. The latter two are primarily used to find the degree of polymerization, the molecular configuration and molecular weight of hyaluronic acid.

The normal synovial fluids from the carpal joints of cattle show high hyaluronic acid concentrations, the highest intrinsic and anomalous viscosity, as well as the highest average relative viscosity with the greatest range. The volumes fluctuated from 2 to 10 ml. with an average of 5 ml. Offhand, it seems strange that the range of relative viscosities was so great, from 9 to 10,300, in the fluids from normal cattle joints. However, it is characteristic of highly polymerized hyaluronic acid in synovial fluid that minimal dilution is followed by great decreases in relative viscosity. Similar wide variations in the range of relative viscosity have been reported for normal human synovial fluid.

The synovial fluid from the carpal joints of the horses had a lower degree of polymerization, as evidenced by the lower intrinsic and anomalous viscosity, and a lower range and average relative viscosity, although the volumes of fluid in both species were similar.

The lower relative viscosity of the horse synovial fluid as compared to the cattle fluid is assumed to be due partly to a lower concentration of hyaluronic acid and partly to a lesser degree of polymerization. While both the cattle and horse joints appeared to be grossly normal, the synovial membrane of the horses showed hyperemia and edema on microscopic examination. Age and species differences may also account for the differences in concentration and polymerization of the hyaluronic acid.

Pathological effusions from human knee joints showed larger volumes than did the normal joints. The decrease in relative viscosity can, therefore, be considered as due in part to dilution. Effusions from osteoarthritis (degenerative) had the largest range and the highest average relative viscosity due both to the greater concentration of hyaluronic acid and the slightly higher mean polymerization.

The rheological findings in osteoarthritis reflect the pathological findings. In this disease destruction of synovial membrane is minimal or absent, inflammatory changes are secondary, and frequently there is villous hypertrophy. This hypertrophy makes possible increased production of hyaluronic acid even though the degree of polymerization is less than normal.

In rheumatoid arthritis the hyaluronic acid concentration, relative viscosity, intrinsic viscosity, and anomaly factor are the lowest for all types studied. No evidence of enzymatic degradation has been found to account for the low polymerization. Therefore, defective synthesis of the hyaluronic acid by the markedly inflamed, infiltrated, and partly eroded synovial membrane is a more likely explanation.

In chronic traumatic synovitis the relative viscosity and the hyaluronic acid concentrations are as low as in rheumatoid arthritis. However, the intrinsic viscosity and the anomaly factor are slightly higher. These findings indicate that the chronic irritation which follows minor trauma leads to marked dysfunction of the synovial membrane. If further investigation confirms these findings it will be of considerable diagnostic value, since objective laboratory, clinical and roentgenographic findings are often insufficient in traumatic synovitis.

For the differential diagnosis of the various types of joint diseases, a combination of the different rheologic findings in synovial effusions provides valuable, although limited, information. For example, synovial fluid with a high relative and anomalous viscosity is more likely to be associated with osteoarthritis than with rheumatoid arthritis or chronic traumatic synovitis.

Sundblad (3) tends to minimize the value of relative viscosity in the differential diagnosis of various types of arthritis. However, from our results it appears to be as valuable as any other single viscosity determination.

For clinical studies the simpler methods of determination of relative and anomalous viscosity which do not require hyaluronic acid determinations should be sufficient.

Viscosity measurement is only one aspect of a systematic examination of synovial effusions which includes cytological, chemical and bacteriological tests as described by Kling (1), Ropes and Bauer (2) and by Sundblad (3). Comprehensive study of the joint effusions combined with a thorough clinical, laboratory and roentgen study are necessary for an adequate differential diagnosis.

SUMMARY

1. A simple capillary viscometer is described, which permits the determination of relative, intrinsic and anomalous viscosity of small volumes of synovial fluid.

2. The various viscosity measurements were determined in synovial fluid from normal carpal joints of horses and cattle and from pathological effusions in human knee joints damaged by osteoarthritis, rheumatoid arthritis, and chronic traumatic synovitis.

3. Hyaluronic acid concentration on these fluids were carried out by a controlled turbidimetric method.

4. Normal synovial fluid from the carpus joint of cattle gave the highest average values for the relative viscosity, intrinsic viscosity and anomaly factor. The concentration of hyaluronic acid was high. The fluid from horses showed viscosity values which were lower than those found in cattle, and a lower hyaluronic acid concentration. The volume of fluid was small in both species compared to pathological human fluid.

5. In the pathological fluids, deviations were most marked in rheumatoid arthritis, less so in chronic traumatic synovitis, and least marked in osteoarthritis.

6. Interpretation of the factors responsible for the differences in viscous behavior are presented.

7. In conjunction with other tests the rheological behavior in normal and pathological fluids provides valuable information about the physiology and pathology of joints.

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BINDING OF CORTICOSTEROIDS BY PLASMA PROTEINS. I. DIALYSIS EQUILIBRIUM AND RENAL CLEARANCE STUDIES [1, 2]

By WILLIAM H. DAUGHADAY, with the technical assistance of RITA BREMER and CAROLYN HARTNETT COLLINS

(From the Department of Internal Medicine, Washington University School of Medicine, St. Louis, Missouri)

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Many biologically significant substances circulate in the plasma bound to plasma proteins to a variable extent. This phenomenon is particularly evident in the plasma transport of certain hormones. The high degree of binding of thyroxine by an alpha globulin is well known. Estrogens, too, have been recognized to have a high affinity for plasma proteins. Bischoff and Katherman (2) observed that estrone was more than 100 times more soluble in human serum than in neutral buffers, a finding indicative of protein binding. A systematic study of the solubilities of certain steroids in bovine serum albumin has been made by Eik-Nes, Schellman, Lumry and Samuels (3). Of the steroids tested, estradiol, Δ 4 androstene-3-one, and androstane-17-one were most tightly bound to bovine albumin serum on the basis of solubility studies. These compounds proved to be more than 400 times more soluble in 5 per cent albumin than in neutral buffers. The affinity of cortisone for albumin was considerably less, and this steroid was only two to three times more soluble in 5 per cent albumin solutions than in buffer. The other steroids of biologic interest exhibited intermediate affinity for albumin. The strength of the steroid protein-union decreased as the number of polar groups of the steroid increased.

Additional observations on the binding of testosterone by bovine serum albumin have been made by Schellman, Lumry and Samuels (4) using dialysis equilibrium and solvent partition analysis in a two-phase system. A linear relation between the reciprocal of the average number of molecules of steroid bound per protein molecule and the reciprocal of the substrate concentration was observed. Differences in the binding affinity of three samples of bovine albumin were attributed to differences in the purity of the proteins. The binding of testosterone increased with temperature and pH, and was inhibited in the presence of thiocyanate ion, methyl orange and zinc.

In this paper the results of dialysis equilibrium experiments with plasma from normal subjects receiving cortisone are presented. The binding affinities of isolated human plasma protein fractions for hydrocortisone are described, and the renal clearance of 17-OH corticosteroids and their glucuronides is interpreted in terms of the physical state of these substances in plasma.

METHODS

Normal adult male volunteers were permitted a low-fat breakfast. Adequate urine flow was achieved by water loading and urine was collected by voiding in thirty-minute periods. Heparinized blood was obtained at the mid-point of the urine collection period. In certain experiments cortisone acetate (75 mg. by mouth) was given after the measurement of endogenous corticosteroid clearances and two hours prior to the collection of urine, for measuring the clearance after loading. Heparinized plasma obtained at the mid-point of this collection was used both to determine renal clearance and also for measurement of binding by dialysis equilibrium. The clearances were repeated in 5 subjects several days later after the ingestion of 2.0 to 2.5 gm. of probenecid.

The binding of 17-OH corticosteroids (17-OHCS) and 17-OH corticosteroid glucuronides (17-OHCS-Gluc) by plasma proteins was determined by dialysis equilibrium in the following manner. One volume of plasma, usually 20 ml., was placed in a Visking cellophane tube (Nojax 18/32 Casing) containing 6 to 10 glass beads. The cellophane bag was placed in a large test tube containing four volumes of Krebs phosphosaline buffer (5). This buffer has a pH of 7.4, an ionic strength of 0.139 and contains the following ions expressed as milliequivalents per liter: Na 158, K 4.6, Mg 4.6, Cl 123, SO_4 4.6,

[1] Reported in part at the Annual Meeting of the American Society of Biological Chemists, April, 1956 (1).

[2] This investigation was supported by a research grant, C-255 (C3), from the National Institute of Arthritis and Metabolic Diseases, of the National Institutes of Health, Public Health Service.

($HPO_4 + H_2PO_4$) 40. The tube was sealed and rotated on a disk 12 times a minute at 4° C for 48 hours. The contents of the bag and the outside dialysis buffer were analyzed for 17-OHCS and 17-OHCS-Gluc. The protein content within the bag was determined by the Weichselbaum modification of the biuret reaction (6).

The method of Nelson and Samuels (7) for plasma corticosteroids has been modified in these studies. Plasma proteins were precipitated with four volumes of acetone and removed by filtration. Fats were removed by partitioning between Skelly Solve B and 80 per cent aqueous acetone. After removal of acetone under reduced pressure, the aqueous phase was extracted with 50 ml. of ethyl acetate three times. Fifteen milliliters of water was used to back-extract the ethyl acetate extract before evaporation to a residue under reduced pressure at 40° C.

17-OHCS-Gluc were determined on the pooled aqueous phases after hydrolysis with bacterial β-glucuronidase (Sigma). Several schedules of addition of enzyme have been investigated to achieve maximum hydrolysis of the glucuronide conjugates. The method selected for use in these studies employed the addition of 800 units of enzyme at two-hour intervals three times, followed by incubation overnight. The liberated steroids were then extracted and the same hydrolysis was repeated on a second day and again extracted with ethyl acetate. The need for such an elaborate scheme of hydrolysis was indicated by analysis of the first and second days' extracts separately. The Porter-Silber chromogen on the second day yielded an additional 20 to 50 per cent.

17-OHCS and 17-OHCS-Gluc were determined in urine and in the dialysate in the equilibrium experiments under the same conditions used for plasma, except that deproteinization and delipidization were not carried out.

Florisil chromatography was performed according to Nelson and Samuels (7) to reduce the nonspecific Porter-Silber chromogens. After suitable corrections for reagent chromogens, sulfuric acid chromogens and the losses of 17-OH corticosteroids on the columns, the concentrations of 17-OHCS and 17-OHCS-Gluc were calculated and expressed in terms of hydrocortisone.

In some clearance experiments urinary 17-OHCS and 17-OHCS-Gluc were measured also by the method of Silber and Porter (8) using 800 units of bacterial beta glucuronidase for 2 ml. of urine and incubating for 24 hours to determine the combined free and glucuronide 17-OHCS.

The human plasma protein fractions (Cohn) were obtained from Dr. J. Newton Ashworth of the American Red Cross. Four per cent protein solutions by weight were prepared and insoluble material was removed by centrifugation. Cellophane bags containing 10 ml. of protein solution were dialyzed against 40 ml. of phosphosaline buffer to which hydrocortisone had been added in dilute alcoholic solution. The final alcohol concentration was 3 per cent at the highest steroid concentrations tested. After 24 hours of dialysis the hydrocortisone in the bag and in the dialysate was measured by the method of Silber and Porter (8). A slight reaction with phenylhydrazine reagent was observed with extracts from albumin solutions to which no hydrocortisone had been added. In experiments with low concentrations of added hydrocortisone, this chromogen of doubtful specificity was subtracted from that obtained in the presence of hydrocortisone. The addition of hydrocortisone to the protein solution rather than to the external buffer resulted in the same steroid distribution, indicating that equilibrium had been closely approached with this period of dialysis.

Creatinine was determined in plasma and urine by a spectrophotometric modification of the method of Folin and Wu (9).

RESULTS

The degree of binding of the plasma 17-OHCS and 17-OHCS-Gluc as indicated by dialysis equilibrium measurements has been summarized in Table I. The concentration of the non-protein bound moiety of the two types of steroids per 100 ml. of water within the dialysis bag has been calculated from the concentration of the steroid outside the bag and the protein concentration within the bag. The concentration of the protein bound 17-OHCS and 17-OHCS-Gluc within the bag is the difference between the total amount of the respective steroids measured within the bag less the calculated free steroid concentration. Under the experimental conditions employed, 94 per cent of the 17-OHCS present within the dialysis bag was bound to the plasma proteins. Although conjugation of 17-OHCS to glucuronides substantially reduced the degree of binding by protein, about two thirds remained associated with the protein components of the system.

The identity of the plasma proteins which are responsible for binding corticosteroids has been investigated by performing dialysis equilibrium stud-

TABLE I

Binding of corticosteroids by plasma proteins *

Expt.	17 OHCS μg./100 ml.		17 OHCS-Glucuronides μg./100 ml.	
	Free	Bound	Free	Bound
1	2.06	29.2	5.15	12.4
2	0.93	29.1	7.46	15.5
3	2.12	39.3	10.0	17.5
4	1.49	26.2	13.3	29.0
5	2.63	30.3	14.0	18.1
Mean	1.85	30.8	10.0	18.5
%	5.7	94.3	35.2	64.8

* Dialysis equilibrium: plasma from normal subjects 2 hrs. after 75 mg. oral cortisone acetate dialyzed against phosphosaline buffer pH 7.4.

TABLE II

Binding of hydrocortisone by plasma protein fractions *

Fractions (Cohn)	Protein mg./ml.	Hydrocortisone Free μg./ml.	Hydrocortisone Bound μg./ml.
I	30	123	12
II	29	113	24
III	19	115	20
IV-1	17	113	1
IV-4	29	103	74
V	30	95	110

* Dialysis equilibrium: human plasma protein fractions and hydrocortisone.

ies using plasma protein fractions prepared by the Cohn method. From the results listed in Table II, the greatest binding activity is evident in the albumin fraction (Fraction V). The affinity of Fraction IV-4 can be ascribed to the albumin content of this fraction. The affinity of the proteins for steroids at this relatively high steroid concentration is less than observed in human plasma. The influence of steroid concentration on the binding of hydrocortisone by human Fraction V and by bovine plasma albumin is illustrated in Figure 1. To compare the affinity of hydrocortisone for albumin the expression $r/[S] = K(n - r)$ has been employed by Eik-Nes, Schellman, Lumry, and Samuels (3). [S] is the molar concentration of unbound steroid and r is the average number of substrate molecules bound per molecule of protein. K is the intrinsic association constant for the binding sites, and n is a function of the binding sites per protein molecule. Under the conditions employed in this study where the value of r is small, r/[S] provides an indication of the magnitude of K for comparative purposes. At steroid concentration of 60 μM per liter, and higher, the affinity was found to be similar to that reported (3). At lower concentrations where the value of r was only a small fraction of the albumin molecules, there was a rise in the value of $r/[S] \times 10^{-4}$. A similar affinity at low hydrocortisone concentration was observed in four other human Fraction V preparations obtained from American Red Cross. One of these human Fraction V preparations did not contain added caprylate.

The process of glomerular filtration represents a physiologic ultrafiltration. It is of interest, therefore, to correlate the *in vitro* dialysis equilibrium experiments which have been carried out with the clearance of 17-OHCS and 17-OHCS-Gluc. The measured clearances of endogenous creatinine, 17-OHCS and 17-OHCS-Gluc are listed in Table III. Although the clearance of 17-OHCS is consistently low, in keeping with the findings of other workers, we have found the clearance of the 17-OHCS-Gluc to be higher in some individuals than that previously reported (10, 11). The variability of the 17-OHCS-Gluc, except in the subject E. J., could not be attributed to obvious analytic errors. Performance of the urinary 17-OHCS-Gluc by two different methods in the last three studies tabulated did not alter the clearances materially. Figure 2 presents the ratios of steroid to creatinine clearance for both the conjugated and unconjugated 17-OHCS, under the four experimental conditions studied. The lower marking tab to the right of the columns indicates the clearance of 17-OHCS predicted on the basis of the dialysis equilibrium experiments assuming that glomerular filtration is the only renal excretory mechanism. The agreement between predicted and observed clearances supports this concept. In the case of the 17-OHCS-Gluc the predicted clearance (upper marking tab) proved to be lower than the observed clearances in the ab-

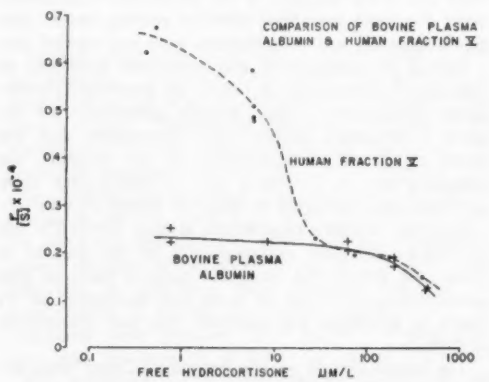

FIG. 1. THE AFFINITY OF HUMAN AND BOVINE ALBUMIN FOR HYDROCORTISONE AT DIFFERENT STEROID CONCENTRATIONS

The albumin solutions were made up to be 4 per cent by weight in phosphosaline buffer. The free hydrocortisone concentration was the concentration of steroid within the bag not in combination with protein. The affinity of albumin for hydrocortisone has been plotted on the ordinate as $r/[S] \times 10^{-4}$. The use of this expression is described in the text.

TABLE III

The Clearance of 17-OHCS and 17-OHCS-Glucuronides

Subject	Cortisone Loading	Benemid	Creatinine Clearance ml/min	17-OHCS Clearance ml/min	17-OHCS-Gluc Clearance ml/min
W.D.	0	0	119	3.5	175
	+	0	115	12.0	156
	0	+	102	15.6	69
	+	+	100	7.1	60
E.J.	0	0	147	7.9	51
	+	0	117	10.9	51
	0	+	112	7.2	100
	+	+	106	14.9	80
W.M	0	0	126	2.9	179
	+	0	148	3.4	192
	0	+	118	3.0	28
	+	+	128	2.4	62
M.L.	0	0	112	9.7	85
	+	0	91	7.6	74
	0	+	95	9.0	51
	+	+	115	8.6	47
S.G.	0	0	125	8.0	76
	+	0	99	8.0	82
	0	+	105	3.2	57
	+	+	106	8.0	43
J.M.	+	0	122	15.4	182
				(12.9)*	(228)*
C.A.	+	0	102	14.1	108
				(12.7)*	(112)*
W.D.	+		119	9.4	76.9
				(9.4)*	(65.8)*

*Urine steroid determinations performed by method of Silber and Porter.

sence of probenecid. This discrepancy suggested a tubular excretory process. It should be noted that tubular excretion of methyl glucuronide, phenyl glucuronide and resorcinyl glucuronide, but not pregnanediol glucuronide, has been demonstrated in the chicken (12). The clearances were repeated following the administration of probenecid and in all except one subject there was a lowering of the 17-OHCS-Gluc clearances in the presence of the drug. The steroid analyses in this case were technically unsatisfactory but have been included for completeness. Probenecid had no effect on the clearance of 17-OHCS. This finding is in agreement with that of Gardner, Crigler, and Migeon (13), who observed that the drug did not alter the excretion of unhydrolyzed 11-oxycorticosteroids although 17-ketosteroid excretion was impaired.

DISCUSSION

The evidence presented indicates that human plasma protein has a high affinity for 17-OH corticosteroids when these steroids are present in physiologic concentrations. The binding is of a loose, freely reversible character. The addition of an organic solvent phase to the dialysis equilibrium system, such as carried out by Axelrod and Zaffaroni (14), results in nearly complete extraction of the corticosteroids from the plasma. Precipitation of proteins with the usual protein precipitants disrupts the steroid-protein union. The nature of the steroid-protein bond is incompletely understood. The participation of the unsaturated ketone at C3 has been suggested by Westphal (15) on the basis of a decrease in the extinction coefficient and a displacement of the absorption maximum in the ultraviolet region of the spectrum of progesterone in albumin solutions. The decreased binding of the 17-OHCS-Gluc might be due both to the hydrophilic character of the glucuronide component and reduction of the unsaturated ketone.

The binding of 17-OHCS restricts the ready

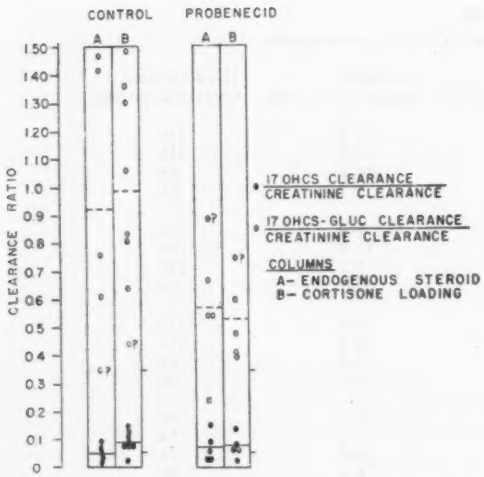

FIG. 2. THE CLEARANCE RATIOS OF 17-OHCS AND 17-OHCS-GLUC WITHOUT STEROID LOADING (COLUMN A) AND AFTER THE INGESTION OF 75 MG. OF CORTISONE (COLUMN B)

At a later date the clearances were repeated after probenecid. The tabs to the right of the columns indicate the clearance predicted on the basis of the simple filtration of non-protein bound 17-OHCS (lower tab) and non-protein bound 17-OHCS-Gluc (upper tab).

passage of corticosteroids across capillary membranes. Evidence for this view may be found in the low concentrations of 17-OHCS which have been reported in normal cerebrospinal fluid (16). The higher concentration of 17-OHCS detected in certain transudates and exudates can be attributed to the protein content of these fluids and the affinity of the respective protein elements for corticosteroids. Ascitic fluid has been tested in this laboratory and has demonstrated affinity for corticosteroids only somewhat lower than plasma. The conclusion of Cope, Hurlock, and Sewell (17) that adrenal cortical hormone diffuses freely into ascitic, pleural, amniotic and synovial fluids needs qualification. It would seem more correct to state that the non-protein bound steroid of the plasma diffuses readily into such fluids and the eventual concentration achieved in such fluids is determined in part by the binding affinity of the proteins of the fluid.

The importance of serum albumin in plasma binding of steroids is indicated by this study. Although considerable denaturation of the lipoprotein fractions occurs with lyophilization, there is no evidence in the present results or in electrophoretic studies presented in an accompanying paper (18) that lipoproteins are important in steroid binding. It is of interest that the affinity of human albumin (Fraction V) for hydrocortisone is greater than bovine serum albumin at lower steroid concentrations. This observation is also in accordance with that of Westphal (15), who observed a greater decrease in ultraviolet extinction when progesterone was bound by human albumin as compared with bovine serum albumin.

The increased affinity of Fraction V for hydrocortisone at low steroid concentrations occurs at steroid to protein molecular ratios far below unity. To explain this behavior it is necessary to postulate that there is a heterogeneity of binding sites either intrinsic or induced by denaturation among albumin molecules, or that the steroid binding sites are partially occupied by other substances in the Fraction V preparations. Although we have demonstrated a high affinity of human Fraction V for hydrocortisone, the degree of binding demonstrable in plasma from patients receiving cortisone exceeds that which can be explained on the basis of the results with Fraction V. In other experiments we have added tracer quantities of hydrocortisone-4-C^{14} to plasma of patients receiving hydrocortisone by mouth to determine whether the binding to plasma proteins which occurs *in vivo* differs from that occurring *in vitro*. The degree of binding was of the same order of magnitude whether measured radiochemically or chemically. The behavior of human albumin at physiologic hydrocortisone concentrations is being studied further.

SUMMARY

1. Dialysis equilibrium experiments have been carried out on human plasma obtained 2 hours after the ingestion of oral cortisone acetate. An average of 94 per cent of the 17-OH corticosteroids and 65 per cent of the 17-OH corticosteroid glucuronides were loosely bound to plasma proteins.

2. Human plasma Fraction V and to a lesser extent Fraction IV-4 possessed significant steroid binding activity. The binding observed in Fraction IV-4 could be attributed to its contained albumin.

3. The binding affinity of human Fraction V increased as the concentration of hydrocortisone decreased. This behavior was not noted in bovine serum albumin.

4. The low renal clearance of 17-OH corticosteroids after cortisone acetate ingestion can be related to low concentration of non-protein bound steroid available for filtration. The clearance of 17-OH corticosteroid glucuronides is higher than can be explained by simple filtration of the non-protein bound component. This observation plus the depression of the clearance of the glucuronide fraction with probenecid suggest a tubular excretory process.

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BINDING OF CORTICOSTEROIDS BY PLASMA PROTEINS. II. PAPER ELECTROPHORESIS AND EQUILIBRIUM PAPER ELECTROPHORESIS [1, 2]

By WILLIAM H. DAUGHADAY

(From the Department of Internal Medicine, Washington University School of Medicine, St. Louis, Missouri)

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Dialysis equilibrium experiments indicate that corticosteroids circulate largely in loose association with plasma proteins (2). When separate fractions of human plasma prepared by the low temperature, alcohol method of Cohn were subjected to dialysis equilibrium against phosphate buffer containing hydrocortisone, Fraction V, largely albumin, proved to have the highest affinity for hydrocortisone.

Additional evidence concerning the nature of the plasma proteins responsible for binding of corticosteroids would be desirable. Paper electrophoresis has been used successfully to characterize the binding of thyroxine (3). An alpha globulin was found to possess the greatest affinity for thyroxine although albumin also may be important in thyroxine binding under certain circumstances. An attempt to apply paper electrophoretic methods to the study of corticosteroid binding has been made by Westphal, Firschein, and Pearce (4). Hydrocortisone-4-C^{14} was added to human serum albumin and subjected to electrophoresis. After development of the electrophoresis the steroid was eluted in strips from the paper and the quantity of radioactivity measured. No migration of hydrocortisone from the starting line was observed. When the same procedure was applied to serum albumin to which progesterone-4-C^{14} had been added, there was a migration of the progesterone with the current, but the steroid was not localized to the albumin component.

In this paper, electrophoretic studies of binding of corticosterone and hydrocortisone by plasma proteins are presented. The standard paper electrophoretic method has been compared with a new method which makes possible the study of the binding of uncharged substances with low affinity for proteins.

METHODS

Sheets of Whatman No. 3 MM filter paper, 18¾ by 9½ inches, were used for electrophoresis between glass plates, as described by Chernoff (5). The avoidance of evaporation such as occurs in most free strip electrophoretic devices is important for binding studies. The edges of the glass plates were sealed with tape to inhibit evaporation further. Barbital buffer pH 8.8 with an ionic strength of 0.06 was used. Electrophoresis was carried out with a current of 180 to 220 volts for a period of from 8 to 14 hours.

Crystalline bovine plasma albumin was obtained from Armour Laboratories. Samples of human Fraction V were provided by Dr. J. Newton Ashworth of the American Red Cross. Blood was drawn from normal human adult laboratory workers and the plasma separated promptly. The Endocrinology Study Section of the National Institutes of Health allotted the corticosterone-4-C^{14} and hydrocortisone-4-C^{14} each with a specific activity of 1.467 microcuries per millimole.

In most experiments 0.2 ml. of the protein solution containing the radioactive steroid was applied to a starting line of 4 to 6 inches in length. After completion of the electrophoresis, the paper was dried in an oven at 100° C, and the position of the proteins was identified by staining with bromphenol blue. Vertical strips, 0.5 inch in width, were cut from the paper. Each strip was then further cut up into small pieces and placed in a test tube. The steroids were extracted from the paper with three 5-ml. portions of ethyl alcohol. The alcohol extract was filtered, pooled and evaporated in 50 ml. conical centrifuge tubes. Radioactivity was determined by transferring the extracts in ethyl alcohol to brass cups with an inside diameter of 2 cm. and containing a disk of lens paper, and drying under an infra red lamp. A Robinson gas flow proportional counter was used to measure the radioactivity. Where a measure of the absolute rather than the relative radioactivity was desired, the transfer was accomplished in 0.6 ml. of 80 per cent ethyl alcohol containing 2 per cent glucose. The glucose fixed the lens paper to the brass cup and provided a uniform glaze

[1] Reported in part at the Annual Meeting of the American Society of Biological Chemists, April, 1956 (1).

[2] This investigation was supported by a research grant, C-255(C3), from the National Institute of Arthritis and Metabolic Diseases, of the National Institutes of Health, Public Health Service.

FIG. 1A FIG. 1B

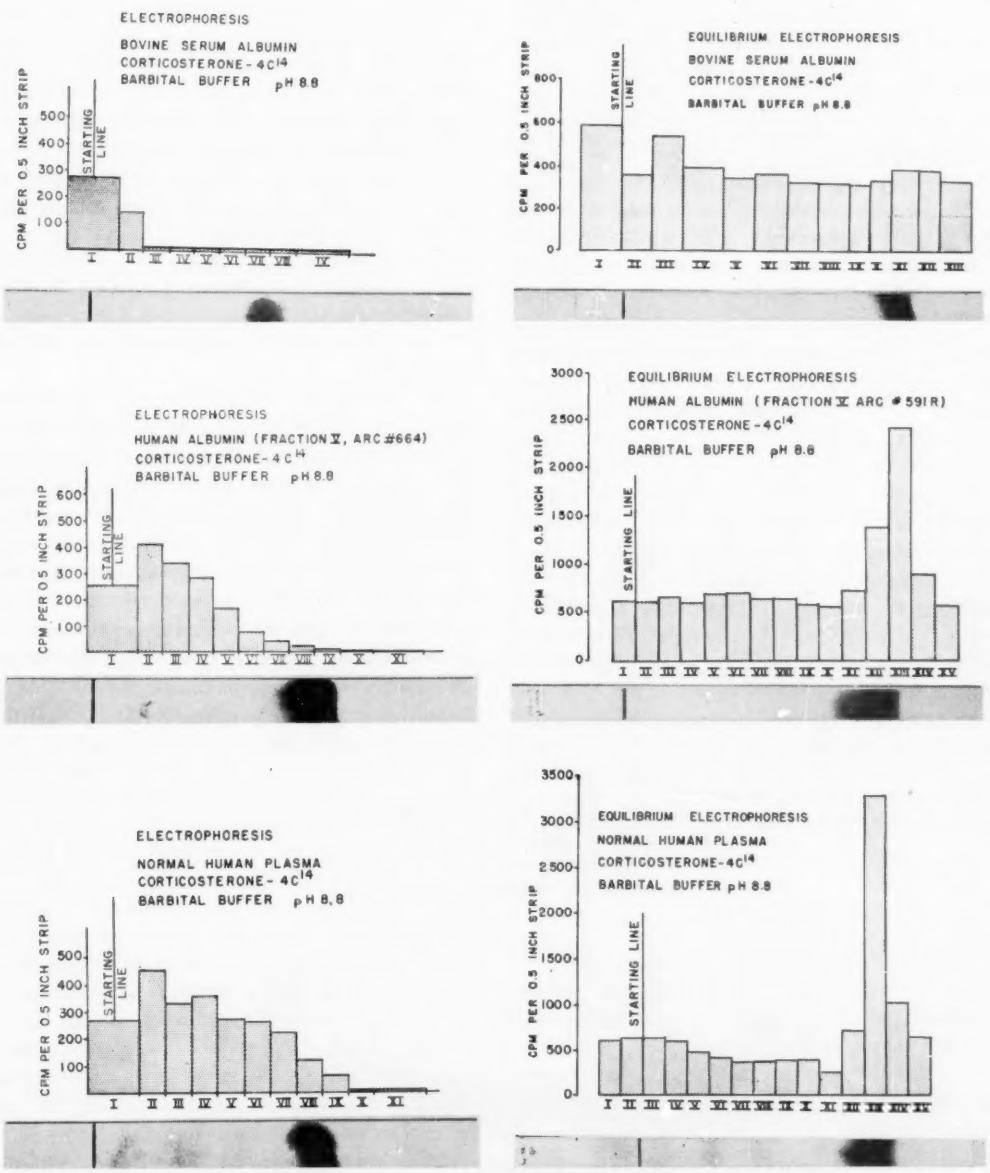

FIG. 1. CONVENTIONAL PAPER ELECTROPHORESIS (1A) AND EQUILIBRIUM PAPER ELECTROPHORESIS (1B) OF BOVINE SERUM ALBUMIN (UPPER GRAPHS), HUMAN ALBUMIN (MIDDLE GRAPHS) AND NORMAL HUMAN PLASMA (LOWER GRAPHS)

The stippled columns indicate the relative amounts of radioactivity found in strips cut vertically to the axis of electrophoresis. Below each graph a strip of paper stained with bromphenol blue indicates the location of the albumin component. The albumin concentrations were 4 per cent except in the case of equilibrium electrophoresis with human albumin (Fraction V) where an 8 per cent solution was used.

after drying under an infra red light. Correction for self absorption was calculated on the basis of weight from an experimentally determined self-absorption curve.

In the technique of equilibrium paper electrophoresis, preliminary equilibrium of the protein solution and the electrophoretic buffer was achieved. The plasma or protein solution, 10 ml. in a cellophane bag (Visking, 18/32 Nojax Casing), was dialyzed for 24 hours against 40 ml. of barbital buffer containing about 20 mcg. of C^{14}-labelled steroid. At the conclusion of the dialysis the paper used for electrophoresis was soaked with the dialysate, and the protein solution was applied to the starting line as described above.

RESULTS

Corticosterone-4-C^{14} was added to bovine plasma albumin, human albumin (Fraction V) and normal human plasma and subjected to paper electrophoresis in the conventional manner (Figure 1A). Significant migration of the radioactivity did not occur with bovine plasma albumin. More of the corticosterone moved in the presence of human albumin, but the greatest movement of corticosterone occurred with normal human plasma. Even with normal human plasma, however, the radioactivity was not associated with a particular protein peak but decreased in a progressive manner from the starting line. In control experiments with corticosterone-4-C^{14}, in the absence of protein there was no obvious migration of steroid from the starting line.

The failure to demonstrate the association of radioactivity with particular proteins in the conventional electrophoresis technic was attributed to the loose and reversible character of the steroid protein bond. As the steroid binding protein advances along the paper a continuous dissociation from the protein occurs with loss of steroid from the moving protein component. The technic of equilibrium paper electrophoresis was designed to maintain the protein in the same free steroid environment that existed during the preliminary dialysis. Under the conditions of equilibrium electrophoresis the rate of association of new steroid with the protein should approximate the rate of dissociation.

The binding of corticosterone-4-C^{14} by bovine and human albumin and by normal human plasma has been examined with the technic of equilibrium electrophoresis (Figure 1B). There was little concentration of radioactivity with the protein peak of bovine albumin. Increase in the protein concentration from 4 to 8 per cent did not improve resolution. In contrast, there was clear localization of corticosterone with the migrating component of human albumin. An even greater localization of radioactivity with the albumin of normal human plasma could be demonstrated.

Parallel experiments have been carried out

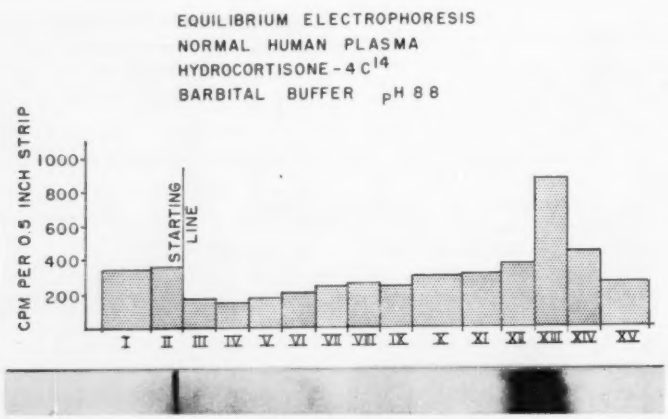

FIG. 2. EQUILIBRIUM PAPER ELECTROPHORESIS OF NORMAL HUMAN PLASMA AND HYDROCORTISONE-4-C^{14}

The stippled columns indicate the radioactivity found in strips cut vertically to the axis of electrophoresis. A strip of paper stained with bromphenol blue indicates the position of the albumin component.

with hydrocortisone-4-C^{14}, and again no meaningful results were obtained with standard paper electrophoretic methods. The binding of hydrocortisone by the albumin component of normal human plasma could be demonstrated clearly by equilibrium electrophoresis (Figure 2). The localization with hydrocortisone was less clearly evident than with corticosterone. This finding could be correlated with the fact that the affinity of corticosterone for albumin is more than twice that of hydrocortisone, as determined by direct radiochemical measurement of the corticosterone-4-C^{14} in the protein solution and the dialysate.

DISCUSSION

The limitations of paper electrophoresis in the study of protein binding of substances such as corticosteroids with relatively low affinity for proteins have been clearly demonstrated. Because of the dissociation of the steroid from the protein, localization with individual protein components cannot be obtained. Westphal, Firschein, and Pearce (4) have inquired whether the greater paper electrophoretic migration of progesterone as compared to hydrocortisone in albumin solution could be attributed to a greater affinity of the hydrocortisone to the paper. Chromatography with barbital buffer actually showed that the reverse was true. The strength of the steroid-protein bond was therefore paramount in determining mobility in paper electrophoresis. The present findings indicate that the major factor promoting dissociation of the steroid is the buffer rather than the paper. Impregnating the paper with buffer containing the corticosteroid in equilibrium with human plasma has permitted continued migration of increased steroid concentration with the albumin component of plasma. It would seem unlikely that the minute concentration of steroid in the buffer could saturate the binding sites for steroid in the paper. Furthermore, when the concentration of steroid was highest in the buffer, as in the runs with bovine albumin, the definition of binding was much poorer than with human plasma when the steroid concentration in the buffer was lower. It may be concluded that the equilibrium between corticosteroid, buffer and protein is not greatly disturbed by the presence of the filter paper.

A graded affinity for corticosteroids has been demonstrated in the proteins studied. Normal human plasma bound the two corticosteroids more strongly than Fraction V. The latter in turn was more potent than bovine plasma albumin. The binding affinity of the last protein was so little that the localization was barely demonstrable by equilibrium electrophoresis. Direct measurement of the radioactivity in protein samples and dialysate prior to electrophoresis demonstrated that the affinity of normal human plasma for hydrocortisone was about eight times greater than that of bovine plasma albumin at the pH of the barbital buffer. The present observations on the differences in the affinity of different albumins for steroids confirm those obtained by dialysis equilibrium (2) and by spectrophotometric studies of binding of progesterone (6). The explanation for these differences cannot be given as yet. There is no evidence of inhomogeneity of the albumin by electrophoresis but it is possible that there is a separate steroid binding protein migrating with the human albumin component. It is possible that the differences between bovine and human albumin may have been induced by fractionation. The close correspondence of the peak of steroid concentration with the albumin component argues against the possibility that the diffuse lipoprotein component which extends from the β-globulins into and sometimes beyond albumin on paper electrophoresis (7) is responsible for protein binding of corticosteroids.

SUMMARY

1. Standard paper electrophoresis failed to indicate the association of corticosterone-4-C^{14} with bovine plasma albumin, human albumin (Fraction V) or with any separate protein component of normal human plasma.

2. Preliminary dialysis of the protein solution to be studied with the electrophoretic buffer containing either corticosterone-4-C^{14} or hydrocortisone-4-C^{14} has allowed the demonstration of the electrophoretic migration of the bound corticosteroid exclusively with the albumin component of normal plasma.

3. The affinity of corticosterone-4-C^{14} and hydrocortisone-4-C^{14} was greatest for normal human

plasma, intermediate for human albumin (Fraction V), and least for bovine plasma albumin.

ACKNOWLEDGMENTS

The author is indebted to Mrs. Carolyn Hartnett Collins for technical assistance, and to Dr. Amos Chernoff for advice on paper electrophoretic methods.

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1. J. Biol. Chem. *218*:335 (Jan.) 1956.

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2. Smith, H. W.: The Kidney, New York, Oxford University Press, 1951, p. 233.

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3. New York State J. Med. *55*:489 (Feb. 15) 1955.

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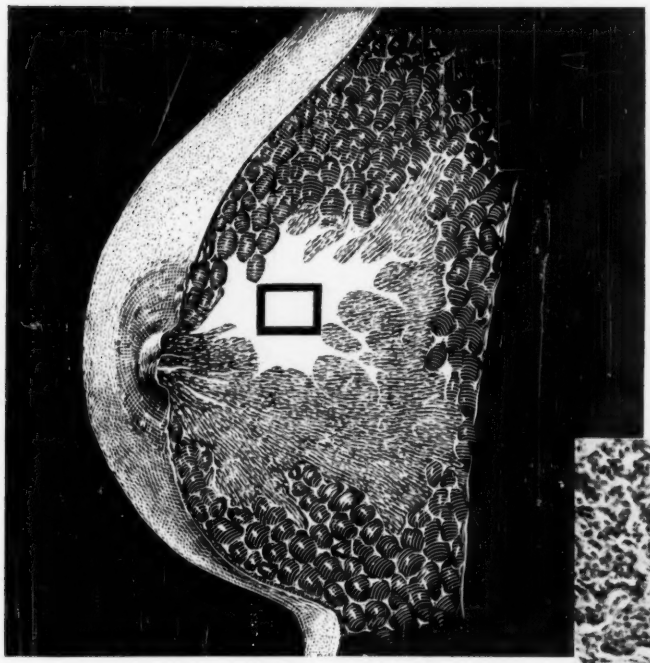

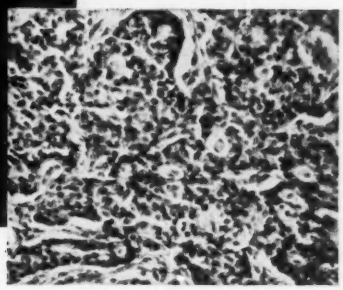

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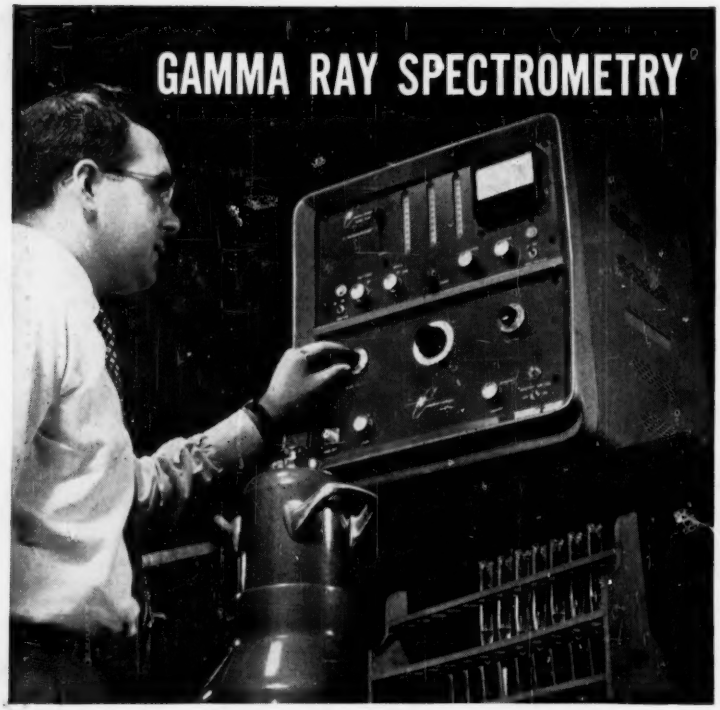

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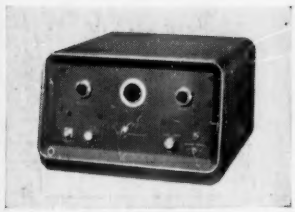

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